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Neural Regions Affecting Female Aggression and Receptivity in *Drosophila melanogaster*

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Graduate Program in Neuroscience

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Abstract

The genetic tools that exist in *Drosophila melanogaster* make it possible to assess the influence of specific regions of the brain on complex behaviour. Examples of such behaviours include female aggression and receptivity to male courtship. Silencing a candidate region called the mushroom body (MB) was found to decrease female receptivity. Additionally, silencing a specific subset of the MB, the alpha/beta lobes, was also found to decrease receptivity. SIFamide neurons are known to affect receptivity, though manipulation of SIFamide signaling in the MB produced no such changes in receptivity. Aggression, another complex behaviour in *Drosophila*, was also affected by genetically controlled neural manipulation. More specifically, hyperactivation of a subset of neurons expressing the *doublesex* gene was found to incite high amounts of aggression in females but not males. Furthermore, the aggression demonstrated by these females differed based on the stimulus presented by the partners, with locomotion being a major elicitor of aggression.

Keywords

Behaviour, sex-specific behaviour, mating behaviour, female receptivity, mushroom body, SIFamide, aggression, grooming, *doublesex*, *Gal4/UAS*, split *Gal4/UAS*, *dTRPA1*, *shibire^{ts1}*, RNA interference.

Summary for Lay Audience

The model organism *Drosophila melanogaster* is highly useful in understanding the neural basis of complex behaviours. Through use of genetic tools unique to *D. melanogaster*, specific areas of the brain can be manipulated to assess their potential roles in facilitating behaviour. This thesis uses this strategy to explore the influence of various brain regions on female responses to partner flies, namely courtship receptivity and aggression.

Manipulation of an area of the brain called the Mushroom Body was found to produce reductions in female receptivity to courtship. Further assessment of this region revealed that manipulation of specific sub regions within the Mushroom Body (the alpha/beta lobes and the alpha prime/beta prime lobes) are also sufficient to influence female receptivity.

Additionally, manipulation of a small group of neurons expressing the *doublesex* gene was found to incite high levels of aggression in females, but not in males. This demonstrates that in *D. melanogaster*, the neural circuitry controlling aggression may be different between males and females. Interestingly, the aggression demonstrated by these transgenic females is variable depending on the nature of the partner fly. For example, less aggression is directed toward female partners than male partners. In an attempt to understand why males received more aggression, new partner types were introduced to the aggressive females. Females which were transgenically made to move more frequently received much higher amounts of aggression than wildtype females. This implies movement is an important stimulus for inciting the observed female aggression. However, the aggressive females also demonstrate aggression toward immobile headless males, meaning movement is not the exclusive cause of aggression. Headless males may incite aggression through chemical stimuli, though this remains to be confirmed.

This is the first known identification of neurons affecting female specific aggression and will provide a novel avenue for exploring this poorly understood behaviour. Additionally, the observation that aggressive behaviours demonstrated are contextually specific provides new considerations for designing future experiments.

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1 Introduction

The neural basis of complex behaviour, though often studied, remains poorly understood. This thesis contains experiments that serve to further the understanding of specific behaviours in the model organism *Drosophila melanogaster*. This first chapter aims to provide better insight into this model, the behaviours of interest to this thesis, as well as the neural and genetic underpinnings of these behaviours.

1.1 Behaviour

The term “behaviour” describes a specific response to stimuli that is not governed exclusively by development (Levitis et al. 2009). The exclusion of developmental phenomena is necessary to differentiate certain internal stimuli. For example, the inflating of *Drosophila* wings is a developmental process, and thus would not technically be considered a behaviour. Response-inducing stimuli can be received from both internal and external sources, and often inform an organism of important biological needs or dangers requiring action. For example, internal stimuli such as hunger sensation may promote foraging behaviours, while external stimuli in the form of seasonal temperature change may promote nesting behaviours. Important external stimuli are also often provided from members of one’s population - responses to stimuli of this nature would be considered social behaviours. Stimuli gathered from one individual that is used by another to adjust its own behaviour can be described as a social cue (reviewed in Danchin et al. 2004). Evolutionarily, it is often beneficial for individuals within a population to exist in a group (Levitis et al. 2009). One potential reason for this group advantage is the ability for individuals to observe others’ social cues and adjust their own behaviours accordingly. For example, female *Drosophila melanogaster* will decrease the number of eggs laid after encountering a parasitoid wasp, which infect larvae. Pairing females who had been exposed to a wasp with unexposed females incites the same change in egg laying behaviour in females who had not been exposed (Kacsoh et al. 2015). Similarly, some social cues communicate information relevant to processes such as reproduction and resource competition (Dukas 2005). Social cues can be conveyed and interpreted in a number of different means and contexts, and can convey a range of information, including threats of immediate danger, mate selection, habitat choice,

resource procuring, and many more. An established model organism for studying the genetic and neural basis of social behaviour is *Drosophila melanogaster* (Greenspan 1995; Nilsen et al. 2004; Ramdya et al. 2017).

1.2 *Drosophila* As a Model For Social Behaviour

1.2.1 *Drosophila* As a Model Organism

Drosophila melanogaster, more commonly referred to as the fruit fly, has been a staple model organism for the study of genetics, development, disease, and behaviour for several decades (reviewed in: Morgan et al. 2013; Hales et al. 2015). *Drosophila* are exceptionally well suited to these areas of research for a number of reasons. First, life cycle and generation times are remarkably quick and females have reasonably high fecundity (Hales et al. 2015). These two traits in tandem allow for comparatively convenient timelines for use in many biological contexts. Manipulation of *Drosophila* genetics is also comparatively easy due to a wide breadth of genetic tools (Beckingham et al. 2005; Stephenson and Metcalfe 2013; Hales et al. 2015). *Drosophila* are also well suited as a model due to evolutionary conservation, including conservation of neural gene function (Lye and Chtarbanova 2018). Additionally, several behaviours exhibited by *Drosophila* are well characterized, allowing for strong informed comparisons when assessing the behaviour of mutants (Nilsen et al. 2004; Ferveur 2010).

1.2.2 *Drosophila* Neurobiology

Although humans and *Drosophila melanogaster* are quite diverged, there remains many similarities between their nervous systems that make *Drosophila* a usable model for understanding nervous system function (Lye and Chtarbanova 2018). Gross general structure is conserved, with the *Drosophila* protocerebrum, deutocerebrum and tritocerebrum being roughly analogous to the forebrain, midbrain, and hindbrain in humans (O’Kane 2011). Additionally, many of the cell types present within the human brain (neurons and various glial cells) also exist within *Drosophila*, and many of them perform the same roles as they do in humans (Freeman and Doherty 2006; Sokolowski 2010). However, mammalian and insect brains are not identical. For example, there are substantially fewer cells in the brains of

Drosophila than in human brains – humans have roughly 100 billion cells, while *Drosophila* brains contain roughly 130,000 (Kaiser 2015). Yet the comparative simplicity of the *Drosophila* brain (though by no means are *Drosophila* simple) may actually be another advantage in using it as a model – understanding a simpler system is more tractable, and can provide an entry point towards informing the workings of more complex organisms.

1.2.3 *Drosophila* Social Behaviour

Drosophila do not rear their offspring or partake in division of labor behaviours, and as a result are considered parasocial. Although *Drosophila* is not technically defined as a fully social species, they do demonstrate a wide variety of social behaviours, including group foraging, aggregation, social learning, and aggression (Nilsen et al. 2004; Tinette et al. 2004; Simon et al. 2012; Kacsoh et al. 2015). Of particular interest to this thesis are female social interactions, which are generally less well-studied than male social interactions. More specifically, this thesis will assess the influence of specific brain regions on two distinct behaviours: female receptivity to male courtship and female aggression. This assessment entails the manipulation of activity within specific subsets of neurons, and the subsequent observation and quantification of behaviour. Manipulation of specific brain regions is made possible through the use of several invaluable genetic tools that exist in *Drosophila melanogaster*.

1.3 *Drosophila* Tools

One of the reasons *Drosophila melanogaster* has become a useful model is the development of many invaluable genetic tools. These tools are often highly versatile, and thus can be used in a variety of contexts to address many different scientific questions. For the purposes of this section, I will focus on tools necessary to understand the experiments described in this thesis. One such tool is the *Gal4/UAS* system, and a recent refinement of this technology, the split-*Gal4/UAS* system.

1.3.1 The *Gal4/UAS* & Split-*Gal4/UAS* System

The binary *Gal4/UAS* system is a two-component system that allows for the tissue-specific expression of a transgene of interest (reviewed in Martín et al. 2017). The first component of

this system, *Gal4*, is a transcription factor endogenous to yeast, but not present within wildtype *Drosophila*. When the *Gal4* gene is placed into the *Drosophila* genome via *P*-element insertion, expression of the *Gal4* gene will occur dependent on nearby enhancer regions. Different insertion sites will allow the *Gal4* gene to be acted upon by different enhancer regions, and thus be expressed in a different anatomical pattern. Therefore, careful selection of enhancer regions controlling the expression of *Gal4* allows for tissue-specific expression of *Gal4*. *UAS*, or Upstream Activating Sequence, is the second component of this system, and acts much like an enhancer region, but requires the presence of the *Gal4* protein in order to initiate transcription of the adjacent gene. Only when *Gal4* is present will the *UAS* be bound and activated, and thus anatomical and temporal expression of the *UAS*-adjacent gene is also defined by the enhancer regions acting on *Gal4*. In this system, transgenics are often created that pair the *UAS* with a gene of interest. Pairing both the *Gal4* and *UAS* components in a single individual then allows for the tissue-specific expression of the *UAS*-paired transgene.

The *Gal4/UAS* system is one of the most powerful tools in *Drosophila* biology - it has been and continues to be incredibly useful in a variety of contexts. However, it does have its limitations, including that the expression of *Gal4* does not always provide a perfect representation of the anatomical region of interest (Martín and Alcorta 2017). A recent refinement of this technology called the split- *Gal4/UAS* system aims to rectify this problem.

The split-*Gal4/UAS* system was developed by Luan et al. in 2006 and allows for more refined anatomical targeting through the use of two enhancer regions each expressing half of the *Gal4* protein. *Gal4* contains two functional domains: a DNA-binding domain responsible for latching the protein onto the DNA strand, and an activation domain responsible for beginning transcription of the *UAS*-adjacent gene. Sequences for these two domains were split and each combined with sequences coding leucine zippers to later facilitate binding of the halves to form a full *Gal4* protein. Each individual domain and its associated zipper are referred to as a hemidriver. Both hemidrivers can then be placed under the control of enhancer regions, much like the binary *Gal4/UAS* system, however different enhancers can be chosen for each hemidriver. Fully functional *Gal4* protein will only be present in cells which have both chosen enhancers active – much like the center of a Venn diagram (Luan et al. 2010). Cells expressing one but not both of the hemidrivers will not contain functioning

Gal4 and thus no *UAS* activation or transgene expression will occur. Use of two enhancer regions allows for very specific areas of expression, occasionally as small as single cells. These refined expression patterns not only provide insight into more specific anatomical regions, but also reduce concern for off-target effects possible when using the binary *Gal4/UAS* system.

1.3.2 Neural Effector Proteins

To assess how specific regions of the brain may influence receptivity and aggression behaviours in *Drosophila melanogaster*, the *Gal4/UAS* system and split-*Gal4/UAS* system can be used to express proteins that alter neuron function in a tissue-specific manner. The proteins *shibire^{ts1}* and *dTRPA1* can be used to silence and hyper-activate neurons respectively in a temperature specific manner. Use of the *Gal4/UAS* system in tandem with *shibire^{ts1}* and *dTRPA1* allows for non-invasive spatial and temporal control of neural activity manipulation.

shibire is a *Drosophila* gene that codes for dynamin, a protein essential for the process of vesicle endocytosis and subsequent recycling (Gonzalez-Bellido et al. 2009). *shibire^{ts1}* codes for a temperature sensitive version of the dynamin protein that becomes non-functional at temperatures above 30°C (Bengtson and Kitamoto 2001). In the absence of functional dynamin, vesicles required for neurotransmitter release cannot be endocytosed or recycled, which culminates in the neuron being unable to release neurotransmitters, and thus being silenced (incapable of communicating with post-synaptic neurons). Return of an individual to permissive temperatures allows temperature sensitive dynamin to return to a functional configuration, allowing normal vesicle recycling and neurotransmitter release (Gonzalez-Bellido et al. 2009).

dTRPA1 (*Drosophila* transient receptor potential cation channel A1) codes for a temperature sensitive Ca^{2+} channel used for heat nociception (Sakai et al. 2009). When exposed to restrictive temperatures above 25°C, the *dTRPA1* protein channel opens, allowing an influx of Ca^{2+} to the neuron, depolarizing it and inciting an action potential (Hamada et al. 2008; Berni et al. 2010). Prolonged exposure to restrictive temperatures will cause the *dTRPA1* ion channel to remain open, continuously causing action potentials – thus neurons under these conditions are considered hyper-active. Much like *shibire^{ts1}*, returning an individual

experiencing *dTRPA1* mediated neural hyper-activation to permissive temperatures will return them to a normal state, as the *dTRPA1* ion channel closes preventing further influx of Ca^{2+} and halting the more frequent action potentials.

1.3.3 RNAi Knockdown

Another strategy that exists in attempting to understand the neural basis of behaviour is to manipulate the presence of specific endogenous proteins through the use of whole organism gene knockouts or tissue-specific gene knockdowns. Whole organism knockouts ensure a gene is completely non-functional, however this may not always be best experimentally - genes may have effects outside of the behavioural context in which they're studied and thus knockouts may produce confounding side effects (Fedorov et al. 2006). Additionally, whole-organism knockout studies cannot always inform which specific tissues underlie the trait of interest. In an effort to circumvent these limitations, techniques have been developed that keep DNA intact while preventing its mRNA product from producing protein. These techniques are collectively referred to as RNA interference (RNAi), and include micro-RNA (miRNA), short hairpin RNA (shRNA), and short interfering RNA (siRNA). Each of these molecules serve to degrade DNA of identical sequence, but differ in their sites of origin and processing to final product (reviewed in Torrecilla et al. 2014).

Of particular interest to this thesis is shRNA interference, and thus its specific means of action will be discussed below. Differences in the means of action of shRNA, miRNA, and siRNA pertain mostly to the start point for the molecules production (miRNA gene *vs* shRNA viral vector), and the proteins required to get from the molecular start point to an RNAi-capable finished product (Drosha and Pasha are required for miRNA processing, while the processing of shRNA requires only Drosha; reviewed in: Torrecilla et al. 2014).

Genes encoding an shRNA molecule contain a complimentary palindromic region, such that after transcription the RNA will fold in half, forming a double-stranded RNA molecule resembling a hairpin (Torrecilla et al. 2014). Hairpin products are processed by the protein Drosha to produce pre-shRNA molecules. Pre-shRNA is then transported out of the nucleus, and subsequently acted on by the protein Dicer to produce shRNA molecules. The now RNAi-capable shRNA are incorporated into the protein complex RISC, which uses the

antisense strand of the shRNA to guide its binding and subsequent cleaving of complementary mRNA. Design of the shRNA transgene such that it is complementary to a gene of interest allows for sequence specific targeting and degradation of the gene of interest's mRNA. This ultimately results in the prevention or reduction of functional gene product of the chosen target gene (Torrecilla et al. 2014). Combining this technology with the previously described *Gal4/UAS* system allows for the tissue-specific knockdown of a specific protein.

1.4 *Drosophila* Courtship and Receptivity

1.4.1 The *Drosophila* Courtship Ritual

Courtship and receptivity are critical in *Drosophila* for propagating an individual's genes. In *Drosophila*, although males initiate courtship, females decide whether or not to proceed with copulation (Greenspan 1995; Villella and Hall 2008), as is often the case when the female of the species has more resource invested in reproduction. Therefore, the responsibility falls on *Drosophila* males to use courtship behaviours to gain the affections of a female and be allowed to copulate. Females evaluate the courtship ritual to infer the quality of a male and his genes, as well as ensuring he is a member of the same species, before permitting copulation to proceed.

Courtship attempts can also occasionally occur between highly diverged species (Gunst et al. 2018), and it is relatively common for *Drosophila* males to court females from closely related species (Carracedo et al. 2000). Mating between species can be maladaptive as a result of the production of unfit offspring (reviewed in: Servedio and Noor 2003). Since females have a greater investment in offspring production, there is particularly strong selection on females to avoid maladaptive matings. Behavioural isolation and sexual selection has resulted in the evolution of species-specific courtship rituals (reviewd in: Laturney and Moehring 2012), and a female's identification of species-specific differences in such rituals serves to prevent maladaptive heterospecific pairings.

Drosophila courtship, though slightly different between species, is a well categorized series of behaviours (Greenspan and Ferveur 2002). Males will initiate courtship by orienting

toward the posterior end of the female, and continuously following the females' movements. He may then present tactile stimuli by tapping the females' abdomen with his forelimb, as well as licking her genitalia. Wing song and chemical cues also provide females with auditory and olfactory stimuli to be evaluated.

Auditory cues conveyed through wing song are composed mostly of single wing vibrations that vary in both frequency and intervals between vibrations. Wing songs are often composed of three distinct alternating sections: a pulse, inter-pulse-interval (IPI), and sine (Kyriacou and Hall 1982). The pulse is a strong beat of the wing, whereas the IPI is the time between adjacent pulses. The duration of IPI are variable between species, and play a major role in a female's determination of song quality (Kyriacou and Hall 1982). After several alternations of pulses and IPI, males may transition to the sine section, also called a "hum," in which a wing is vibrated at a consistent frequency producing a sound much like a sine wave (Greenspan and Ferveur 2002).

Drosophila courtship also involves the transmission of chemical signals, called cuticular hydrocarbons (CHCs). CHCs are nonvolatile and as a result are only communicated when individuals are in close proximity with one another, as would be the case during courtship (Bontonou and Wicker-Thomas 2014). A high degree of variability exists in the CHCs presented by different species of *Drosophila*, making them a key stimulus for identification of conspecifics. There are also sex differences in the CHCs presented by individuals of the same species (Bontonou and Wicker-Thomas 2014).

Should the female's assessment of the courtship ritual identify the male as being of poor quality or belonging to a different species, she will display rejection behaviours such as moving away, ovipositor extensions, kicking, etc. in service of preventing copulation (Cook and Connolly 2008). In contrast, if the stimuli presented are identifiable as a conspecific courtship attempt, and be deemed of sufficient quality, the *Drosophila* female will engage in receptivity behaviours – behaviours allowing or encouraging a male to fertilize her eggs. Such behaviours include pausing, spreading the wings, and opening of the vaginal plates (Ferveur 2010).

1.4.2 Genetic and Neurological Basis of Female Receptivity

Female receptivity to male courtship involves the perception, assessment, and response to a variety of different sensory stimuli, and as a result has a complex genetic and neurological basis. Due to the multiple components involved, it is not surprising that multiple genes and regions of the brain have been shown to affect female receptivity.

Of the several genes implicated in *Drosophila* sexual behaviours, among the most well-studied are *fruitless* (*fru*) and *doublesex* (*dsx*), with *dsx* being of particular importance to female receptivity. Both the *fru* and *dsx* loci are highly complex, having multiple splice variants, some of which are sex-specific and important in the process of sexual differentiation within the sex-determination pathway (Chandler et al. 2003). The sex-specific transcripts of these genes are also expressed extensively within the central nervous system, with specific expression patterns differing between the sexes (Lee et al. 2002; Dickson 2008). These genes play a significant role in the formation of sex-specific behaviour (Rideout et al. 2010; Zhou et al. 2014; Sellami and Veenstra 2015). *fru* has been extensively linked to male sexual behaviours and the development of sex-specific neural circuitry (Demir and Dickson 2005; Dickson 2008), though its roles in female receptivity are much less well understood. One study identified that when silencing a subset of *fru* neurons in females individuals become much less receptive to male courtship (Kvitsiani and Dickson 2006). The role of *dsx* in female receptivity is slightly more informed. Hyper-activation of specific *dsx*-expressing cells resulted in elevated levels of receptivity, while silencing those same neurons produced the opposite effect of reducing receptivity (Zhou et al. 2014).

Besides the frequently studied genes *fru* and *dsx*, other genes have also been shown to influence female receptivity. Neurons expressing *apterous*, a gene also encoding a transcription factor, were found to influence female receptivity (Aranha et al. 2017). These neurons did not co-express either *fru* or *dsx*, indicating that the *apterous* neurons are not a subset of the neurons expressing these sex-determination genes (Aranha et al. 2017). The precise role of *apterous* in modulating receptivity is thought to be a result of changes in female walking speed, as unreceptive females will often walk away from courting males. However, silencing *apterous* neurons also alters female post-mating behaviours, namely egg-laying and ovipositor extrusion (a rejection behaviour) (Aranha et al. 2017). Mutations in the

retained gene cause high amounts of rejection behaviours from females, as well as inciting male-like courtship independent of *fru* neurons (Ditch 2004). Elimination of the *spinster* gene product in brain areas processing chemical and olfactory cues (*spin-A* and *spin-D* clusters) induces high levels of female rejection (Sakurai et al. 2013). *Neuroglian* (also called *icebox*) mutants also differ from wildtype controls in their receptivity, potentially as a result of severe brain developmental defects including the lack of development of mushroom bodies (Carhan et al. 2005). Lastly, the *painless* (Sakai et al. 2009) and *datilograf* (Schinaman et al. 2014) genes have been associated with female receptivity.

Specific neurotransmitters have been functionally linked to female receptivity. Cholinergic and GABAergic neurons are the sites of action for *painless* and *datilograf* mediated changes to receptivity (Sakai et al. 2009; Schinaman et al. 2014). Females lacking dopamine signaling show increased time to copulation (Neckameyer 1998b). Dopamine has also been shown to be necessary for male learning in sexual behaviour contexts (Neckameyer 1998a). Increasing the presence of the neurotransmitter octopamine has been shown to decrease receptivity, partially through action on *dsx* neurons (Rezával et al. 2014a). Additionally, ablation or SIFamide-knockdown of SIFamide-expressing neurons in females have all been shown to incite higher levels of copulation (Terhzaz et al. 2007).

As suggested above, specific regions of the *Drosophila* brain have been shown to influence female receptivity. Perhaps not surprisingly, many of these brain areas are those involved in the processing of sensory information. For example, *retained* mutations, described above, appear to exert their effects on female receptivity through developmental defects in the suboesophageal zone (SEZ) (Ditch 2004). The SEZ is an important brain region for the processing of both gustatory and olfactory processing. (Koganezawa et al. 2010; Kwon et al. 2014). Similarly, the *spin-A* and *spin-D* neuron clusters discussed above for their expression of *spinster* also occupy the SEZ and antennal lobe, respectively (Sakurai et al. 2013). The antennal lobe is known to be extensively involved in olfactory processing, and contains dendrites of the Or47b cluster contributing to the *spinster*-mediated receptivity phenotype (Sakurai et al. 2013). The Johnston's organ and the antennal mechano-sensory and motor center are both highly important for processing of auditory stimulus, and therefore courtship song (Boekhoff-Falk and Eberl 2014), making them strong candidate regions for influencers of female receptivity. Mutations in both *retained* and *neuroglian* cause morphological

anomalies in the mushroom body, suggesting that this brain region may play a role of in female receptivity (Ditch 2004; Carhan et al. 2005). Lastly, visual stimuli are likely unimportant to the receptivity phenotype (Aranha et al. 2017).

The involvement of numerous genes and brain regions demonstrates that female receptivity is a highly complex behaviour. In the interest of advancing the understanding of this process, this thesis aims to assess the roles of two candidate neural sets, namely the mushroom body and SIFamide neurons, on female receptivity. The rationale for focusing on those two neuronal substrates is described below.

1.5 The Mushroom Body

1.5.1 Function & Associated Behaviours

One brain region of particular interest to this thesis shown to be involved in sensory processing and female receptivity is the mushroom body (MB). The mushroom body is a heavily-studied, complex region of the *Drosophila* brain that has been implicated in a variety of behaviors. Most often discussed are its influences on the high order functions of learning and memory (Davis 1993; Mershin et al. 2004). The MB's role in memory can be extended to both short and long term memory (Zars et al. 2000; Dubnau et al. 2001; Pascual and Pr  at 2002), as well as stimuli-specific contextual memory (Vogt et al. 2014), including olfactory learning (De Belle and Heisenberg 1994). Manipulation of the MB has been shown to affect olfactory (De Belle and Heisenberg 1994), gustatory (Masek and Keene 2016) and visual stimuli processing (Vogt et al. 2016). The MB receives input from multiple sensory modalities (Yagi et al. 2016), which makes it a likely site of sensory integration (Davis 1993). As all of these sensory modalities are pertinent to the process of female receptivity, the MB's role in processing these types of information makes it a strong candidate region for an influencer of female receptivity.

Other processes shown to be associated with the MB are sleep regulation (Yi et al. 2013; Kanold et al. 2015), motor function (Martin et al. 1998; Mabuchi et al. 2016), and larval feeding behaviours (Zhao and Campos 2012). Lastly, the MB has also been implicated in

choice behaviours, further strengthening it as a candidate region for female receptivity (Zhang et al. 2007; Solanki et al. 2015).

1.5.2 Anatomy & Physiology

The mushroom body is an anatomically complex region of the brain, consisting of the projections from thousands of cells, called Kenyon cells, and occupying two bilateral halves comprised of a calyx, a pedunculus, and five lobes (Tanaka et al. 2008). The Kenyon cell bodies are located in the cortex above the calyx (Aso et al. 2009). The aggregation of dendrites of the Kenyon cells form the calyx, which acts as a main source of extrinsic neuron input, and can be further subdivided into the main calyx, dorsal accessory calyx, lateral accessory calyx, and ventral accessory calyx (Stocker et al. 1990; Yagi et al. 2016). The axons from the Kenyon cells form the pedunculus and the five MB lobes, with each lobe branching from the anterior end of the pedunculus (Tanaka et al. 2008). Classification of Kenyon cells is largely based on the lobe(s) the axon projects into: gamma, alpha/beta, and alpha prime/beta prime (Ito et al. 1997). Gamma, beta, and beta prime lobes extend horizontally from the pedunculus, while alpha and alpha prime extend vertically. Thus, alpha/beta and alpha prime/beta prime Kenyon cells bifurcate at the end of the pedunculus and extend both vertically and horizontally (Figure 1). The lobes are the main source of output for the MB, but also receive projections, and thus neural input, from other structures (Ito et al. 1998; Tanaka et al. 2008).

The MB lobes have been shown to contain cells with different morphologies and marker expression levels, suggesting functional differences among the lobes (Ito et al. 1998; Strausfeld et al. 2003; Tanaka et al. 2008). For example, the gamma lobes have been shown to contain many cells expressing *fru* (Yu et al. 2010), implying a potential role in sex-specific behaviours. Further complicating the MB's structure is the presence of different cells *within* lobes. For example, three distinct cell types have been identified within the gamma lobes (Mao 2009). Similarly, the alpha/beta and alpha prime/beta prime lobes have been shown to contain different cell subtypes based on the gene expression and neurotransmitters used by these neurons (Strausfeld et al. 2003; Tanaka et al. 2008).

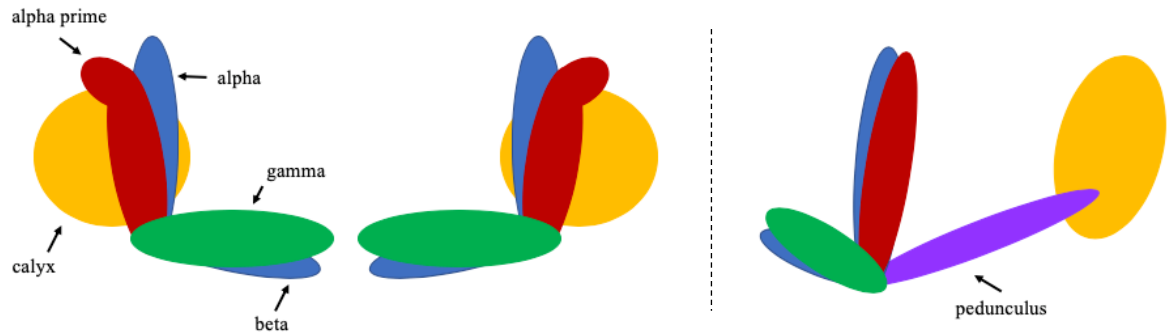


Figure 1. Schematic representation of the Mushroom Body. Anterior view (left, with dorsal end on top) showing the bilateral pair of lobe sets, and lateral view (right) depicting pedunculus connecting the calyx and lobes. Beta prime lobes lay posterior to the gamma and beta lobes and are therefore not visible in schematic.

The anatomical complexity of the MB may in part explain the plethora of associated functions (Aso et al. 2009) and the ability to segregate the processing of different sensory stimuli to specific regions within the structure (Yagi et al. 2016). A recent study supported the hypothesis of functional segregation in the MBs by demonstrating that specific silencing of the gamma lobes, but not other lobes, results in impaired courtship memory in males (Montague and Baker 2016). These add further credibility to the idea that individual MB lobes may act as effectors of sex-specific behaviours, which may include female receptivity.

1.6 SIFamide

Within the *Drosophila* nervous system, the neuropeptide AYRKPPFNGSIFamide (SIFamide) is very poorly understood. Expression of SIFamide is limited to only four neurons occupying the pars intercerebralis (Terhzaz et al. 2007). However, expression of the receptor for SIFamide is widespread throughout the central nervous system (Terhzaz et al. 2007). The pars intercerebralis, which SIFamide neurons occupy, is known to have neuroendocrine function and thus secretes cell products into the hemolymph to be diffused (Taghert and Veenstra 2003). The four SIFamide neurons do not function this way, and instead release SIFamide exclusively synaptically (Terhzaz et al. 2007). This implies that the

four identified SIFamide neurons are highly branched and extend to the various brain areas where the receptor is found.

Though little is known about the function of SIFamide, studies have shown that it may be involved in an array of processes. Ablation of SIFamide neurons, as well as pan-neural knockdown of SIFamide and its receptor, have all been shown to decrease sleep amount (Park et al. 2014). Hyper-activation of SIFamide neurons has also been shown to increase specific feeding behaviours, namely food quantity uptake and approach towards food odorants when satiated (Martelli et al. 2017). SIFamide action on feeding behaviours may act through olfactory projections to the antennal lobe (Martelli et al. 2017).

Of particular interest to this thesis is the influence of SIFamide on sex-specific behaviours. Ablation of SIFamide neurons in males results in indiscriminate courtship behaviours toward both females and males. This result is recapitulated when SIFamide is site-specifically knocked down via RNAi within the four SIFamide neurons (Terhzaz et al. 2007). Performing the same experiment in females produces similar results – females with ablated SIFamide neurons or SIFamide neuron specific knockdown of SIFamide both display decreased time to copulation when compared to genetic controls (Terhzaz et al. 2007). These “promiscuous” females are in contrast to unpublished data produced by the Moehring lab (2018), which showed silencing of SIFamide neurons induces an increase in female sexual rejection behaviours. Experiments conducted in this thesis will attempt to delineate downstream neural regions important for SIFamide-mediated changes to female sexual receptivity.

1.7 *Drosophila* Aggression

1.7.1 Aggressive Behaviour

Aggression is a highly important set of behaviours that is often required for procurement of resources and mates. Aggressive behaviours can be defined as acts of attack or threat. Such behaviours can be directed toward conspecifics or heterospecifics in a variety of contexts: acquisition of food, mates, or territory, and defense of self, offspring, or mates (reviewed in Zwarts et al. 2012). It is important for individuals to demonstrate aggressive behaviours in

the correct context and at the right moment, as becoming aggressive too readily may encourage more frequent dangerous encounters (Zwarts et al. 2012).

Although stereotypically considered to be a male-specific set of behaviours, aggression in females occasionally occurs in *Drosophila*, as well as many other species (Nilsen et al. 2004). However, the contexts in which females become aggressive are slightly different than males. Male *Drosophila* aggression motivations include establishing dominance, earning territory, and obtaining food and mates. Female aggression motivations primarily consist of rejecting male courtship or procuring food; this latter situation occurs more readily if yeast content is high, which is proposed to beneficially affect egg-laying capability (Zwarts et al. 2012). One study identified the influence of male ejaculate on inciting various post-mating female behaviours, which often include aggression (Bath et al. 2017). One proposed motivation for increases in female aggression due to mating is that egg production increases metabolic demand, and thus a heightened need for food resources inspires more aggression. This was shown not to be the case, as post-mating increases in aggression were also observed in females incapable of producing eggs. (Bath et al. 2017). The same study showed that post-mating female aggression is strongly tied to both sperm and male sex peptide in the ejaculate.

The specific behaviours displayed in *Drosophila* aggression have been characterized in both sexes. Many aggressive behaviours are shared between males and females, though behaviours exclusive to one or the other also exist. Of the most common aggressive displays in *Drosophila* are orienting (turning toward opponent), approaching (moving toward opponent), wing extensions (moving one wing out to roughly 90 degrees or both wings out to roughly 45 degrees), and various lunges (thrusts toward the aggressor) (Nilsen et al. 2004; Zwarts et al. 2012). Some of the less frequently used aggressive behaviours include decanting (sudden flight away from the opponent) and fencing (extending the limbs to make contact with the opponent). As mentioned, some sex-specific differences exist in how aggression is conveyed. Competing males will often display boxing behaviour, which has not been observed in females. This involves both males rearing up on hind legs and striking each other with forelimbs (Zwarts et al. 2012). Head-butting is a female-specific variation of lunging (both of which are forms of shoving) which involves thrusting toward the opponent such that the aggressor's head makes contact with the opponent's abdomen (Nilsen et al. 2004).

1.7.2 Genetic and Neurological Basis of Aggression

Although the ethogram of female aggressive behaviours has been characterized, research on the underlying genetic and neural basis of aggression is almost always conducted in males. Due to the differences in the types of aggressive behaviours displayed between males and females, and the different contexts in which females and males may display aggression (Zwarts et al. 2012), it stands to reason that the genetic and neural basis for aggression may be subtly different between the sexes. Therefore, it is useful and necessary to understand female aggression in order to understand aggression as a whole.

Although few in number, experiments have been conducted that have informed the neural underpinnings of female aggressive behaviour. Female flies lacking the neurotransmitter octopamine took longer to begin aggressive demonstrations, and additionally showed fewer attempts at female aggression specific head-butting when compared to genetic controls (Zhou et al. 2008). Targeted expression of octopamine within octopamine mutants rescued this phenotype. Similar effects were also seen in males lacking neural octopamine. Conversely, over-abundance of octopamine was shown to decrease aggression latency and increase bouts of shoving in males (Zhou et al. 2008).

While we know little about the neural basis of female aggression, much more is known about the neural basis of male aggression, and it is possible that some of the neural underpinnings of aggression are shared between males and females though this remains to be seen experimentally (Lee and Hall 2000; Alekseyenko et al. 2013). Several neuropeptides have been shown to influence male aggression, including NPF, TK, and DH44 (reviewed in: Nässel and Zandawala 2019). Hyper-activation of two specific dopaminergic neurons induces high amounts of male aggression. These neurons, T1 and PPM3, contain presynaptic terminals in the protocerebral bridge, tritocerebrum, fan-shaped body, and noduli, potentially pointing to a role in male aggression for each of these regions (Alekseyenko et al. 2013).

Mutations of the *fru* gene have been studied extensively for their effects on male behaviour. Of the behavioural anomalies observed in *fru* mutant males is their willingness to court other males (Ito et al. 2002). A study by Lee and Hall (2000) showed a lesser known *fru* mutant behaviour – aggression-like head interactions. They found that many of the previously

developed homozygous viable mutant *fru* stocks demonstrated a behaviour in which males interact head to head, as opposed to head to tail configurations more common in courtship (Lee and Hall 2000). Preceding the holding of head to head position, *fru* mutant males will extend forelimbs to tap or slash at the opponent/partner, not unlike the fencing behaviours observed in aggressive males. These results may imply the *fru* gene, or the subset of neurons that express this gene, is involved in sex-specific aggression neural circuitry.

1.8 The *doublesex* Gene & Sex-Specific Behaviour

The *dsx* gene is a critical component in the process of sexual differentiation and has been shown to affect development of sex-specific neural circuitry (Lee et al. 2002; Sanders and Arbeitman 2008; Rideout et al. 2010; Pavlou and Goodwin 2013). Both males and females contain three clusters of neurons expressing *dsx*: pC1, pC2, and pCd. *dsx* neurons are less numerous in females and the pC1, pC2, and pCd clusters are smaller. Additionally, male brains contain additional *dsx* expressing neurons not seen in females. However, *dsx* expression is much more abundant in the female ventral nerve cord, specifically in the abdominal ganglia.

Due to the many sex-specific differences in expression, it is not surprising that *dsx* has been shown to affect a number of sex-specific behaviours. Firstly, *dsx* has been implicated in non-aggressive post-mating behaviour. Expression of membrane-bound male sex peptide within *dsx* neurons resulted in virgin females mating at lower frequencies that were similar to mated control females (Rezával et al. 2012). Females of this genotype also demonstrated more frequent rejection behaviours, namely ovipositor extrusions. *dsx* neuron knockdown of the male sex peptide receptor was sufficient to rescue all behavioural phenotypes (Rezával et al. 2012). A group of neurons within the ventral nerve cord expressing both *dsx* and the neurotransmitter octopamine have also been implicated in female post-mating behavioural change (Rezával et al. 2014a). Hyper-activation of abdominal ganglion neurons co-expressing both octopamine and *dsx* produced females that displayed more frequent ovipositor extension and that were less receptive to male courtship. Interestingly, no neurons in the brain co-express both octopamine and *dsx* (Rezával et al. 2014a)

A subset of *dsx* neurons has also been implicated in male aggression. GABA receptor knockdown in a subset of pC1 *dsx* neurons shown not to co-express *fru* resulted in high amounts of male aggression. The same result was achieved through hyper-activation of this same group of neurons (Koganezawa et al. 2016). Interestingly, other cells occupying the pC1 cluster marked positive for *fru* but negative for *dsx* were shown to strongly incite indiscriminate male-male courtship (Koganezawa et al. 2016). These results in combination have led to two *fru* neurons occupying pC1 (LC1 and mAL) to be considered a neural “switch” governing elicitation of courtship or aggression behaviours. Such a switch remains to be identified in females.

Silencing of *dsx* neurons has been shown to decrease female receptivity as well as egg laying (Rideout et al. 2010), while hyperactivation has the opposite effect in enhancing female receptivity (Zhou et al. 2014). Expression of neural effector proteins in various subsets of *dsx* neurons also results in changes to female receptivity behaviours. In 2014, Zhou et al developed new *Gal4* driver lines by pairing the *Gal4* gene with carefully selected non-coding regions of the *dsx* gene shown to have enhancer activity. The result was a group of *Gal4* drivers expressing in sexually dimorphic subsets of *dsx* neurons. These *Gal4* drivers were made to express neural effector proteins to assess the roles of specific *dsx* neurons in sex-specific behaviour. They found that hyper-activation of neurons occupying the pC1 and pCd clusters increased receptivity. Silencing the same neurons had the opposite effect. Additionally, pC1 and pCd were both found to influence female receptivity through the processing of the male pheromone cVA, while only pC1 was found to additionally process male courtship song (Zhou et al. 2014). Expression of neural effector proteins within specific *dsx* neurons occupying the abdominal ganglia has also been shown to influence female receptivity (Jang et al. 2017).

Recently, Andrea Bevan of the Moehring lab conducted experiments assessing the effects of *dsx* neuron clusters on female receptivity, with the goal to repeat some of the above experiments but assessing if the same neurons could increase female receptivity towards heterospecific males. Using the *Gal4* lines developed by Zhou et al. in 2014, she expressed the neural effector protein *dTRPA1* to hyper-activate specific subsets of *dsx* neurons in females. Surprisingly, she observed that hyper-activation of one particular group of *dsx* neurons, defined by the 40F04-*Gal4* driver, induced high levels of female aggression.

Included in this thesis are experiments that aim to provide detailed descriptions of 40F04-Gal4/+;UAS-dTRPA1/+ female aggression, as well as determine the specific contexts in which aggression is induced.

1.9 Summary of Experimental Objectives

The neural basis of female receptivity and aggression remains poorly understood in *Drosophila*. Understanding the neural basis of female receptivity may shed light on the process of evolution on a fine scale. Functional or structural differences in brain regions affecting female receptivity may provide a basis for differences in mate selection criteria between species. Changes in these regions may allow for different sexual selective pressures which can culminate in speciation. The study of female aggression serves to delineate the sex differences in how aggression is displayed, as well as differences in the contexts this important behaviour is elicited. This thesis aims to further our understanding of these social behaviours through site-specific manipulation of brain function.

Different regions of the *Drosophila melanogaster* brain were assessed for their influence on receptivity or aggression in three distinct groups of experiments. First, I aim to assess the role of specific mushroom body lobes on influencing female receptivity. Through use of the *Gal4/UAS* and split-*Gal4/UAS* systems, specific lobes of the MB will be made to express neural effector proteins *dTRPA1* or *shibire^{ts1}*, which will allow for the temperature-dependent hyperactivation or silencing of the targeted lobe. Females with these neural changes will be compared to genetic controls in respect to their receptivity to wildtype virgin conspecific males. Differences highlighted by these comparisons will potentially reveal specific regions of the MB involved in the process of female receptivity. Identification of any such brain region may allow for further functional dissection of the area, through use of alternate split-*Gal4* drivers expressing in more specific areas *within* the identified region.

My second objective is to further delineate the circuitry underlying SIFamide-mediated changes to female receptivity. Much like the first group of experiments, the *Gal4/UAS* system and split-*Gal4/UAS* system will be used to evaluate the influence of specific regions of the female brain. However, in place of neural effector proteins, these experiments will involve the site-specific expression of RNAi designed to knock-down the translation of the

SIFamide receptor protein. This receptor must be present in neurons downstream of SIFamide neurons, and thus removal of the receptor in the causal connecting neurons should induce the same rejection behaviors as silencing the SIFamide neurons themselves. Again, females with these neural changes will be paired with wildtype conspecific males and their receptivity behaviour evaluated. If similar phenotypes to SIFamide silencing are observed in females lacking SIFamide receptors in a specific region, the region expressing the SIFamide RNAi is likely an important downstream region mediating SIFamide's effects on female receptivity. Again, any regions identified by this method may be further functionally dissected using split-*Gal4* drivers expressing in more narrow regions *within* the identified area.

The third and final objective for this thesis is to characterize the aggressive behaviours demonstrated by 40F04-*Gal4/+;UAS-dTRPA1/+* females (females with a subset of *dsx* neurons hyperactivated). To further this characterization, I also attempt to ascertain the aggression-inducing stimuli. 40F04-*Gal4/+;UAS-dTRPA1/+* females and their genetic controls will be paired individually with wildtype conspecific males and the precise amounts of each aggressive behaviour measured. Similar experiments will be conducted using various partner types chosen for their differing social cues (ex. wildtype *Drosophila simulans* males and wildtype *Drosophila melanogaster* females). Comparisons of the amounts of aggressive behaviours directed toward these varying partners will provide understanding of the specific stimuli necessary to incite aggression from 40F04-*Gal4/+;UAS-dTRPA1/+* females. Additionally, a neural mapping experiment will be conducted using the split-*Gal4/UAS* system to confirm that 40F04-*Gal4* neurons are in fact *dsx*-expressing. As the initial identification of this aggression phenotype occurred during experiments assessing female receptivity, 40F04-*Gal4/+;UAS-dTRPA1/+* males have yet to be behaviorally characterized for the phenotypes observed in 40F04-*Gal4/+;UAS-dTRPA1/+* females. As a result, I will also pair 40F04-*Gal4/+;UAS-dTRPA1/+* males with wildtype conspecific males to determine if similar behavioural changes are observed.

2 Methods

2.1 Stocks

“Wildtype” control *Drosophila melanogaster* stock melBJS, collected in London, Ontario in 2009, was provided by Dr. Brent Sinclair. *Drosophila simulans* sim199 (stock # 14021-0251.199) was purchased from the Drosophila Species Stock Center (Cornell, New York). All transgenic lines were purchased from Bloomington Drosophila Stock Center (Bloomington, Indiana) except the *dsxDBD* stock, which was provided by Dr. Stephen Goodwin. Stock #49436 was chosen because of the strong hyper-mobility phenotype identified in the Janelia Fly Bowl project (Simon and Dickinson 2010). All stocks are outlined in Table 1.

2.2 Fly Maintenance and Crosses

All *Drosophila* stocks were housed in 30 mL vials containing standard cornmeal medium (Bloomington *Drosophila* Stock Center recipe). Vials containing flies within one generation of experimentation were housed in a controlled incubator set to 24 °C, ~70% humidity, and a 14:10 light:dark cycle. When not within a generation of experimentation, stocks were kept at room temperature (~24 °C). All crosses were performed using 1-5 males paired with 1-10 virgin females aged 2-10 days old, placed in a new food vial in a 24 °C incubator.

Generation of the split-*Gal4* combination *R40F04-p65.AD-Gal4/+;dsxDBD-Gal4/UAS-dTRPA1* flies involved the only multigenerational crossing scheme used in this thesis (Figure 2). Both 71042 and *dsxDBD* stocks were crossed to 3703, a stock containing multiple balancer chromosomes containing indicator phenotypes (Table 1). Progeny from one cross displaying select indicator phenotypes were then crossed to the appropriate progeny from the other cross. Progeny from second generation cross containing both transgenes were selected and self-mated to produce a stock homozygous for both 71042 and *dsxDBD*. This stock was then crossed to *UAS-dTRPA1* to *R40F04-p65.AD-Gal4/+;dsxDBD-Gal4/UAS-dTRPA1* flies for use in assays (Figure 2).

Table 1. Wildtype and transgenic fly strains.

Wildtype Controls			
Stock Name	Description	Genotype¹	Stock #
melBJS	"wildtype" <i>D.melanogaster</i>	++;+;	n/a
sim199	"wildtype" <i>D. simulans</i>	++;+;	n/a
Gal4 & Split-Gal4 Drivers			
458	<i>elav-Gal4</i> . Expresses in all nervous tissue.	$P\{w^{+mW.hs}=GawB\}elav^{C155}; +; +$	458
49265	<i>rutabaga-Gal4</i> . Expresses in the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=GMR15E01-GAL4\}attP2; +$	49265
MB152	Split- <i>Gal4</i> driver expressing in the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R19B03-p65.AD\}attP40; P\{y^{+7.7} w^{+mC}=R26E07-GAL4.DBD\}attP2$	68266
MB008	Split- <i>Gal4</i> driver expressing in alpha/beta lobes of the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R13F02-p65.AD\}attP40; P\{y^{+7.7} w^{+mC}=R44E04-GAL4.DBD\}attP2$	68291
MB185	Split- <i>Gal4</i> driver expressing in alpha/beta lobes of the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R52H09-p65.AD\}attP40; P\{y^{+7.7} w^{+mC}=R18F09-GAL4.DBD\}attP2$	68267
MB005	Split- <i>Gal4</i> driver expressing in alpha prime/beta prime lobes of the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R13F02-p65.AD\}attP40/CyO; P\{y^{+7.7} w^{+mC}=R34A03-GAL4.DBD\}attP2$	68306
MB461	Split- <i>Gal4</i> driver expressing in alpha prime/beta prime lobes of the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R35B12-p65.AD\}attP40; P\{y^{+7.7} w^{+mC}=R26E07-GAL4.DBD\}attP2$	68327
MB009	Split- <i>Gal4</i> driver expressing in gamma lobes of the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R13F02-p65.AD\}attP40/CyO; P\{y^{+7.7} w^{+mC}=R45H04-GAL4.DBD\}attP2$	68292
MB131	Split- <i>Gal4</i> driver expressing in gamma lobes of the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R13F02-p65.AD\}attP40/CyO; P\{y^{+7.7} w^{+mC}=R89B01-GAL4.DBD\}attP2$	68265
MB419	Split- <i>Gal4</i> driver expressing in a region of the gamma lobes of the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R26E07-p65.AD\}attP40/CyO; P\{y^{+7.7} w^{+mC}=R39A11-GAL4.DBD\}attP2$	68323
MB607	Split- <i>Gal4</i> driver expressing in a region of the gamma lobes of the MB.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R19B03-p65.AD\}attP40; P\{y^{+7.7} w^{+mC}=R39A11-GAL4.DBD\}attP2$	68256
40F04	<i>Gal4</i> driver expressing based on a subset of <i>dsx</i> enhancer regions.	$w^{1118}; +; P\{y^{+7.7} w^{+mC}=GMR40F04-GAL4\}attP2$	50094
49436	<i>Gal4</i> driver. Hyper-activation of these neurons induces hyper-mobility, as seen in <i>Janelia</i> fly bowl (Simon and Dickinson 2010).	$w^{1118}; +; P\{y^{+7.7} w^{+mC}=GMR27G11-GAL4\}attP2$	49436
<i>dsx</i> DBD	Split- <i>Gal4</i> hemidriver expressing <i>Gal4</i> DBD in <i>doublesex</i> neurons.	$+; +; TI\{GAL4(DBD)::Zip\}dsx^{GAL4-DBD}$	n/a
71042	Split- <i>Gal4</i> hemidriver expressing <i>Gal4</i> AD in 40F04 neurons.	$w^{1118}; P\{y^{+7.7} w^{+mC}=R40F04-p65.AD\}attP40; +$	71042

Stock Name	Description	Genotype	Stock #
UAS effectors			
26263	<i>UAS-dTRPA1</i> .	$w^*; P\{y^{+t7.7} w^{+mC}=UAS-TrpA1(B).K\}attP16; +$	26263
44222	<i>UAS-shibire^{ts1}</i> .	$w^*; +; P\{w^{+mC}=UAS-shi^{ts1}.K\}3$	44222
6314	<i>UAS-GFP</i> that expresses in membranes.	$y^l w^* P\{w^{+mC}=UAS-mCD8::GFP.L\}Ptp4E^{LL4} P\{w^{+mW.hs}=GawB\}Iz^{gal4}; +; +$	6314
34947	<i>UAS</i> allows <i>Gal4</i> dependent expression of RNAi targeting SIFamide receptor.	$y^l sc^* v^l sev^{21}; +; P\{y^{+t7.7} v^{+t1.8}=TRiP.HMS00299\}attP2$	34947
Crossing Tools			
3703	Contains balancer chromosomes with visible phenotypic markers.	$w^{1118}/Dp(1;Y)y^+; CyO/nub^1 b^1 sna^{sco} It^1 stw^3; MKRS/TM6B, Tb^1$	3703

¹ Genotypes for the three large *Drosophila* chromosomes are shown, separated by a semi-colon. The genotype of the small 'dot' fourth chromosome is not shown. "+" is wildtype.

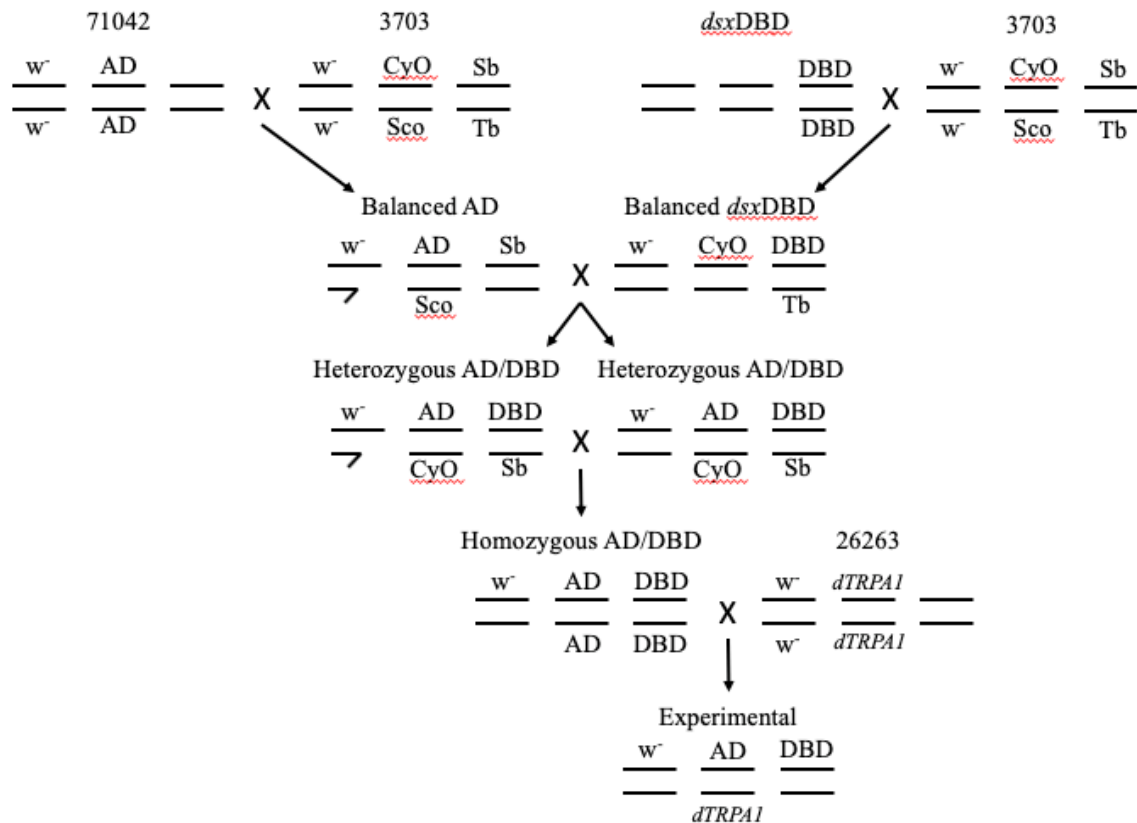


Figure 2. Crossing scheme used to combine 40F04-*Gal4*-AD, *dsxDBD*, and *UAS-dTRPA1* into a single genotype.

2.3 Confirmation of Functional Transgenics

2.3.1 *Gal4* Drivers, Brain Dissection, & Fluorescence

Microscopy

Efficacy and specificity of *Gal4* and split-*Gal4* driver stocks was assessed by crossing driver lines to a *UAS-GFP* (green fluorescent protein) stock, and subsequent confirmation of expected fluorescence patterns (Jenett et al. 2012; Aso et al. 2014). Females aged 4 to 7 days old containing both *UAS-GFP* and the desired driver were anesthetized using CO₂ and decapitated. Heads were washed with 70% ethanol for approximately 2 minutes, at which point they were moved to PBS buffer on a dissection plate. Head cuticle, trachea, and other debris were removed from the brain using microdissection tweezers. Clean brains were then transferred to a microscope slide containing more PBS using a micropipetter. After ensuring brains were in a suitable orientation, a coverslip bridge was constructed using clear nail polish as an adhesive. Fluorescent imagery was conducted on a Nikon Eclipse Ci-L upright fluorescent microscope with an attached DS-Fi2 colour camera. Images were recorded using Nikon Elements D software.

2.3.2 Temperature Sensitive Neural Effector Proteins

Efficacy of temperature sensitive *UAS* neural effector (*dTRPA1* and *shibire^{ts1}*) stocks was assessed through crosses to an *elav-Gal4* driving expression in all nervous tissue. Individuals aged from 4 to 7 days containing both the *elav-Gal4* and the chosen *UAS*-neural effector were placed in an assay chamber, and the assay chamber placed in an incubator set to 30°C and ~70% humidity. Individuals were recorded with an iPad for 40 minutes from the moment they were placed at the restrictive temperature. Videos were later assessed for behavioural change associated with pan-neural expression of active temperature-sensitive neural effector proteins (seizing and paralysis for *dTRPA1* and *shibire^{ts1}*, respectively; Kitamoto 2001; Berni et al. 2010).

2.4 Mating Assay

Courtship assays were conducted in assay chambers developed and generously provided by Dr. Jamie Kramer. Each chamber contains 18 circular isolated assay wells measuring 1 cm in diameter. Each well contains a movable partition dividing it in half and preventing the interaction of flies on either side. The partitions can be removed at will, allowing for temporal control of fly interaction. The chamber is cleaned between assays.

Individual assays involve three virgin female genotypes: one experimental (*Gal4/+;UAS/+*) and two genetic controls (*Gal4/+;+* and *+;UAS/+*), all aged between four and seven days. Assays consistently took place within the first four hours of lights on. Since the entire sample size could not be tested within a single day, and to ensure uniform environmental effects on each genotype (Austin et al. 2014), equal numbers of each female genotype are used within each assay. Each female is aspirated into an individual well with the partition in place. The other half of each well is filled by a wildtype virgin male also aged between four and seven days. The now full assay chamber is placed in a 30° incubator for 30 minutes with the partitions still in place to ensure the activation of temperature-sensitive transgenics before the pairs interact. Following the 30-minute temperature acclimation, video recording begins and the partitions are removed. Interactions are recorded for 30 minutes.

Scoring of mating assay videos includes the identification and recording of two specific behaviours: initiation of courtship and initiation of copulation. Initiation of courtship is scored as the first instance the wildtype male demonstrates clear orienting or following. Copulation start is recorded as the time at which flies can be seen as physically connected in copula for 5 or more minutes. Note that only one case of early termination of copulation (<5 minutes) was observed. Measurement of courtship and copulation start across a number of individuals was used to calculate proportion of copulated females of those courted as well as duration of courtship. Proportion of copulated females and duration of courtship are used as indirect measures of female receptivity, and thus changes in these behaviours will hereafter be referred to as changes in female receptivity. If necessary, recordings can be used to gather other metrics including latency to courtship, proportion of courted females, and male courtship intensity. Differences in the intensity of male courtship are expected, though assumed to be equal across genotypes. Confirmation of consistent male behaviour between

experimental groups can be accomplished through thorough scoring of male behaviours. Only females that were courted were included in the sample size, as females have to first be courted in order to be receptive or rejectionary towards males. Sample sizes following these exclusions are listed in Tables 2 and 3, as well as in figure captions.

2.4.1 Statistical Analysis

Number of copulated females out of those that were courted in treatment and control genotypes was compared using Chi square analysis. Identification of differences between groups was followed by post hoc pair-wise Chi square tests to identify which groups significantly varied from the others. Courtship duration differences between genotypes were assessed using Kaplan Meier survival analysis. Logrank p values indicating differences between genotypes were followed by pairwise Logrank analysis. As multiple hypotheses are being tested, a false discovery rate correction was conducted to ensure statistically significant differences remain genuine despite multiple comparisons.

2.5 Aggression Assay

Aggression assays were conducted very similarly to courtship assays. Experimental (40F04-*Gal4/+;UAS-dTRPA1/+*) and control (40F04-*Gal4/+;+* and *+/+;UAS-dTRPA1/+*) females of equal number aged four to seven days were paired one on one with a partner in the same assay chamber described above. Assay chamber partitions remained within wells during aspiration and a 30-minute acclimation in a 30° incubator. Following the 30-minute acclimation to ensure temperature-sensitive activation of *dTRPA1*, video recording commenced and well partitions were removed to allow female interaction with partner flies. Recordings ran for 10 minutes. Females were paired with: melBJS males, decapitated melBJS males, melBJS females, hyper-locomotive *GMR27G11-Gal4/+;UAS-dTRPA1/+* females, or sim199 males. In the case of assays containing 40F04-*Gal4/+;UAS-dTRPA1/+* females paired with decapitated individuals, flies had been decapitated an hour prior to acclimation to ensure no twitching was present that may cause movement-incited aggression from 40F04-*Gal4/+;UAS-dTRPA1/+* females.

Scoring aggression assay video involved recording instances of aggressive behaviours in experimental and control females. Aggression behaviours were classified as in Nilsen et al. (2004). Tallied aggressive behaviours included orienting, approaching, wing threats, head-butting (shoving), and a group of less common aggressive behaviours deemed “other” which included decanting, lunging, and fencing. The time at which each of these behaviours occurred was recorded, and the total amounts of each behaviour counted. In addition to aggressive behaviours, instances of head grooming were also recorded. More specifically, head grooming frequencies and durations were tallied and calculated using each grooming start time and grooming end time.

2.5.1 Statistical Analysis

Statistical analysis was performed using R studio 1.1.456. For experiments in which control groups contained behaviour tallies of mostly zeros, genotypes were compared using Kruskal Wallis analysis. Identification of statistical difference between groups was followed by a post hoc Dunn test to identify which group varied significantly from the others. Comparison of groups not comprised mostly of zeros, such as total 40F04-*Gal4/+;UAS-dTRPA1/+* female aggression counts toward different partners, was conducted via Poisson regression. Post hoc tests to identify differing groups consisted of modified Tukey tests accommodating the Poisson regression (R package detailed in Hothorn et al. 2008)

3 Results

3.1 Confirmation of Transgenics

In order to make claims regarding which brain areas do or do not influence a particular behaviour, I first confirmed transgenics were functioning as expected. Occasionally, fly stocks may become contaminated resulting in flies lacking the desired transgenic entirely. If this were the case in behavioural experiments, no conclusions regarding the influence of the brain regions of interest could be drawn, as the lack of intended transgenics results in no manipulation of the intended brain region. Here I outline the results of preliminary experiments designed to confirm the presence of the chosen transgenics.

3.1.1 *Gal4* and split-*Gal4* drivers

Gal4 and split-*Gal4* stocks were confirmed to express in expected patterns (Jenett et al. 2012; Aso et al. 2014) by crosses to a *UAS-GFP*, and subsequent brain dissection and fluorescence microscopy of the appropriate F1 progeny (those lacking balancer chromosome indicator phenotypes). Between 3-5 females of each *Gal4* driver;*UAS-GFP* were dissected. I found that all driver lines displayed the expected fluorescence pattern (Figures 3 and 4).

3.1.2 *UAS* neural effectors

UAS-dTRPA1 functionality was assessed by hyperactivation of all nervous tissue using the pan-neural *elav-Gal4* driver. Five *elav-Gal4/+;UAS-dTRPA1/+* females placed in the activating temperature of 30°C demonstrated uncontrollable, erratic movement and seizing after roughly 8 minutes. Normal movement returned to all females upon movement of these individuals back to a permissive room temperature of 24°C.

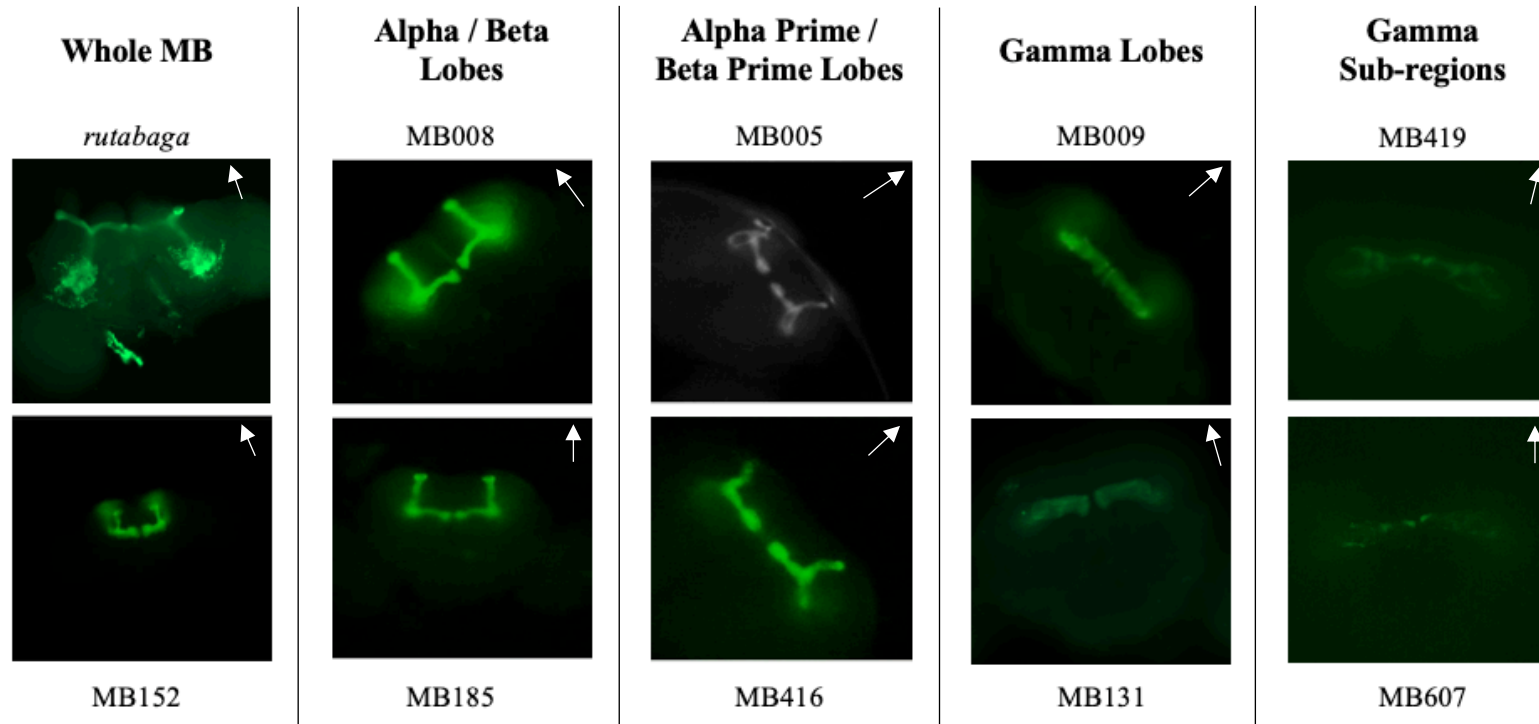


Figure 3. Confirmation of MB *Gal4* and split-*Gal4* driver line efficacy through expression of membrane bound GFP (green fluorescent protein). Drosophila brains transgenically made to express GFP within specific MB lobes. MB anatomical regions are listed in columns, with each brain region tested using two different genetic drivers, as shown. Brain orientation differs between images.

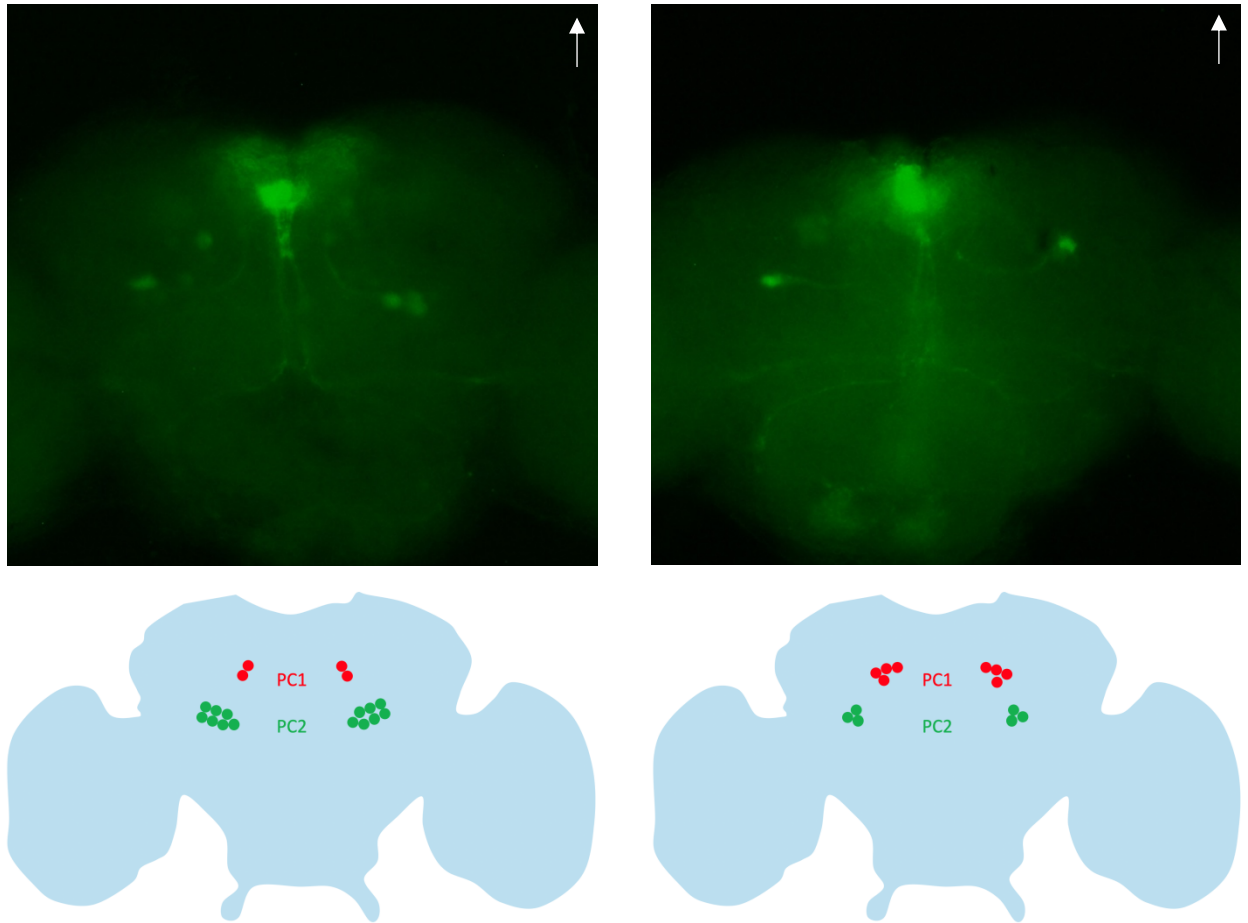


Figure 4. Confirmation of 40F04-*Gal4* driver line efficacy through expression of GFP (top) and schematic representation of expression pattern (bottom) in a female (left) and male (right) brains (image modified from Zhou et al. 2014). *Drosophila* brains transgenically made to express GFP within subsets of pC1 and pC2 neurons. Note that the image used membrane-bound GFP which fluoresces the entire neuron, while the schematic shows only the location of the neuron cell bodies. Additionally, schematics include only *dsx* positive 40F04 cells, while brain images include off target non-*dsx* expressing cells. White arrows denote dorsal end of brain.

Similarly, *UAS-shibire^{ts1}* was also expressed pan-neutrally to ensure efficacy. Fourteen *elav-Gal4/+;UAS-shibire^{ts1}/+* females were placed at the restrictive temperature of 30°C and were observed to have ceased movement as early as 1 minute, but more often after roughly 5 minutes. Perturbing the assay chamber resulted in all treated flies falling and failing to upright themselves, demonstrating complete paralysis. Much like *elav-Gal4/+;UAS-dTRPA1/+* females, all *elav-Gal4/+;UAS-shibire^{ts1}/+* females returned back to normal following movement to a permissive temperature of 24°C.

3.2 Mushroom Body

Following the confirmation of transgenics, I then used various combinations of these lines to assess whether the MB as a whole influences female receptivity. Following these experiments, I then addressed the question of whether manipulation of specific MB lobe subsets are sufficient to influence female receptivity.

3.2.1 Silencing the MB body reduces female receptivity

Silencing MB activity via the expression of *shibire^{ts1}* was found to decrease proportion of copulating females, as well as increase courtship duration. MB specific expression of *shibire^{ts1}* was accomplished using the *rutabaga-Gal4* and MB152 split-*Gal4* driver lines, both of which produced decreases in receptivity. Of the females that were courted, *rutabaga-Gal4/+;UAS-shibire^{ts1}/+* females demonstrated lower proportions of copulation (Figure 5)($\chi^2 = 7.52$, $df = 2$, $p = 0.023$), with post-hoc pairwise comparisons confirming differences between treatment females and both genetic control genotypes. Courtship duration was also shown to be affected in *rutabaga-Gal4;UAS-shibire^{ts1}* females, with treatment females showing longer time to copulation from courtship start (Figure 6)(Kaplan Meir Logrank $p = 0.04$). Pairwise analysis also confirmed courtship duration differences between the treatment and both control genotypes. Much of the same was also shown for MB152 split-*Gal4/+;UAS-shibire^{ts1}/+* females. Lower proportions of MB152 split-*Gal4/+;UAS-shibire^{ts1}/+* females copulated when compared to controls (Figure 7)($\chi^2 = 8.69$, $df = 2$, $p = 0.013$) as confirmed by post-hoc pairwise comparisons. Courtship duration was also shown to be longer in MB152 split-*Gal4/+;UAS-shibire^{ts1}/+* females (Figure 8)(Kaplan Meir Logrank $p = 0.01$). Use of *rutabaga-Gal4* and MB152 split-*Gal4* to express the temperature-sensitive neural-hyperactivation protein *dTRPA1* produced no differences in female receptivity when compared to genetic controls.

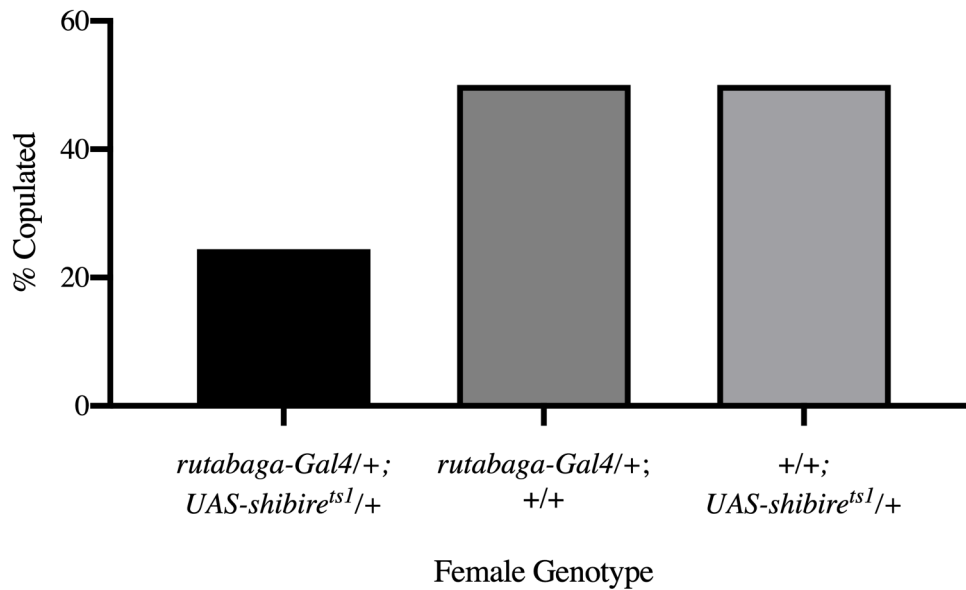


Figure 5. Silencing of the MB via expression of *shibire^{tsl}* by the *rutabaga-Gal4* driver causes a reduced proportion of copulating females ($n = 45/36/36$, $\chi^2 = 7.52$, $df = 2$, $p = 0.01$, pairwise chi vs. split-*Gal4* control $p = 0.017$, pairwise chi vs. *UAS* control $p = 0.017$). Bars represent the percentage of copulated females of those that were courted.

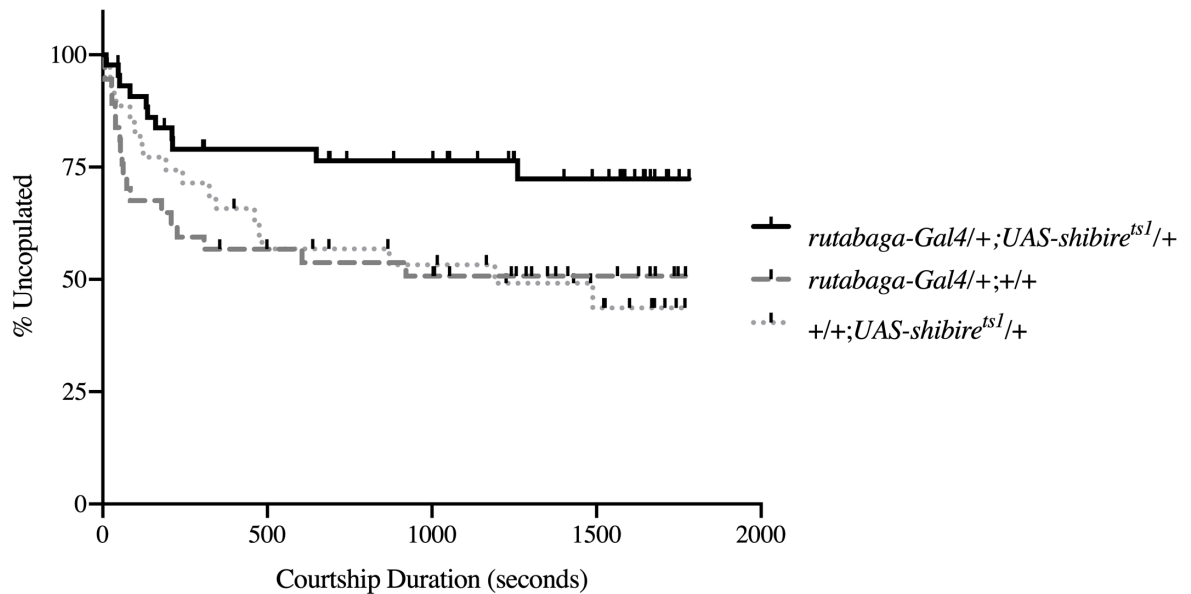


Figure 6. Silencing of the MB via expression of *shibire^{tsl}* by *rutabaga-Gal4* driver causes prolonged courtship duration ($n = 45/36/36$, Kaplan Meier concordance = 0.602, Logrank test $p = 0.03$, pairwise logrank vs. *Gal4* control $p = 0.037$, pairwise logrank vs. *UAS* control $p = 0.037$). Curve represents proportion of copulated females over time. Tick marks denote Kaplan Meier censorship – assay ended before copulation occurred.

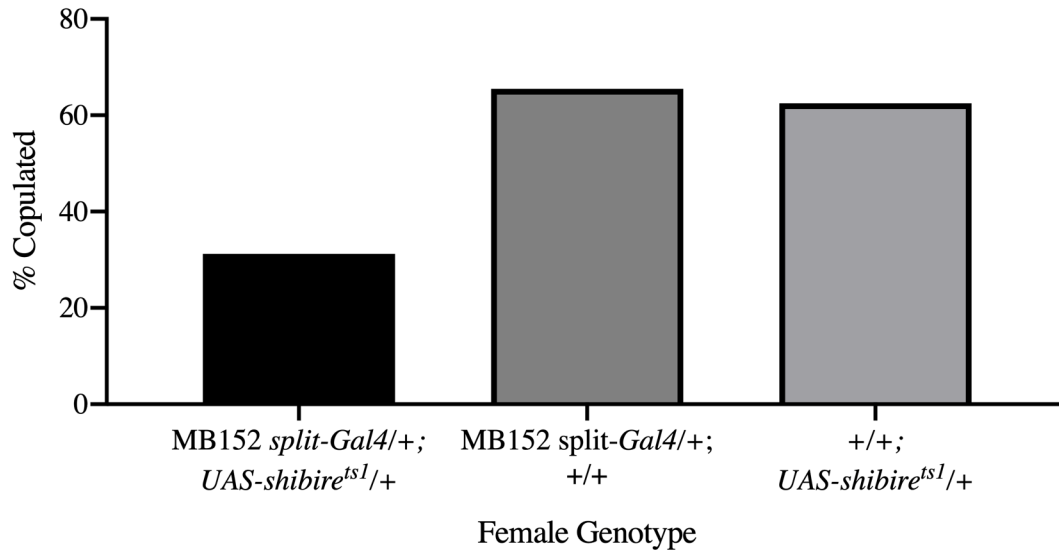


Figure 7. Silencing of the MB via expression of *shibire^{ts1}* by the MB152 *split-Gal4* driver causes a reduced proportion of copulating females ($n = 32/29/24$, $\chi^2 = 8.69$, $df = 2$, $p = 0.02$, pairwise chi vs. *split-Gal4* control $p = 0.007$, pairwise chi vs. *UAS* control $p = 0.0019$). Bars represent the percentage of copulated females of those that were courted.

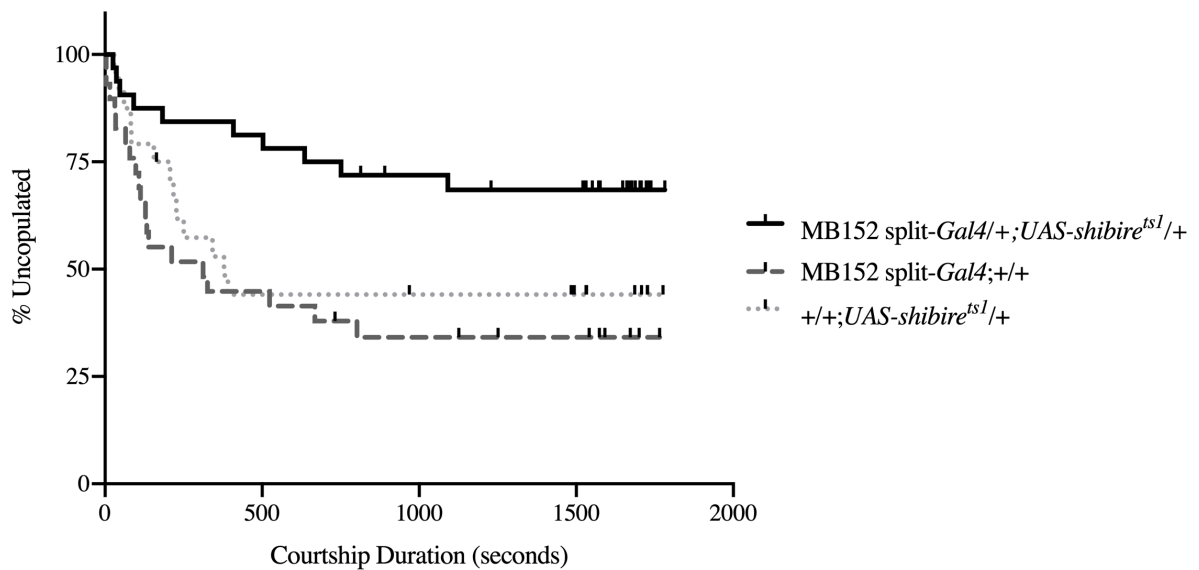


Figure 8. Silencing of the MB via expression of *shibire^{ts1}* by the MB152 *split-Gal4* driver causes prolonged courtship duration ($n = 32/29/24$, Kaplan Meier concordance = 0.631, Logrank test $p = 0.01$, pairwise logrank vs. *Gal4* control $p = 0.011$, pairwise logrank vs. *UAS* control $p = 0.067$). Curve represents proportion of copulated females over time. Tick marks denote Kaplan Meier censorship – assay ended before copulation occurred.

3.2.2 Specific lobe subsets of the MB influence female receptivity

To determine whether subsets of neurons within the MB are influencing female receptivity, I silenced or hyper-activated specific lobes of the MB and assessed the effect on female receptivity. Silencing of the alpha / beta lobes in MB185 split-*Gal4/+;UAS-shibire^{ts1}/+* females produced statistically lower proportions of copulating females after multiple testing correction (Figure 9)($\chi^2 = 12.24$, $df = 2$, $p = 0.002$). Additionally, longer courtship durations were observed in MB185 split-*Gal4/+;UAS-shibire^{ts1}/+* females (Figure 10)(Kaplan Meier Logrank $p = 0.002$). Silencing of the alpha prime/beta prime or gamma lobes within the MB did not produce a significant difference in female receptivity when compared to genetic controls (Table 2).

Hyperactivation of the alpha prime / beta prime lobes in MB461 split-*Gal4/+;UAS-dTRPA1/+* females produced reductions in proportion of copulated females as well as increases in courtship duration. MB461 split-*Gal4/+;UAS-dTRPA1/+* females showed reduced proportions of copulation when compared to genetic controls (Figure 11)($\chi^2 = 7.22$, $df = 2$, $p = 0.02$), as well as longer courtship durations (Figure 12)(Kaplan Meir Logrank $p = 0.03$). Pairwise comparisons showed MB461 split-*Gal4;UAS-dTRPA1* females are distinct from controls in regards to both measures of female receptivity. However, neither of these statistical differences remained significant following false discovery rate correction for multiple testing. Hyperactivation of several other regions of the MB did not produce any notable differences in female receptivity when compared to controls (Table 3).

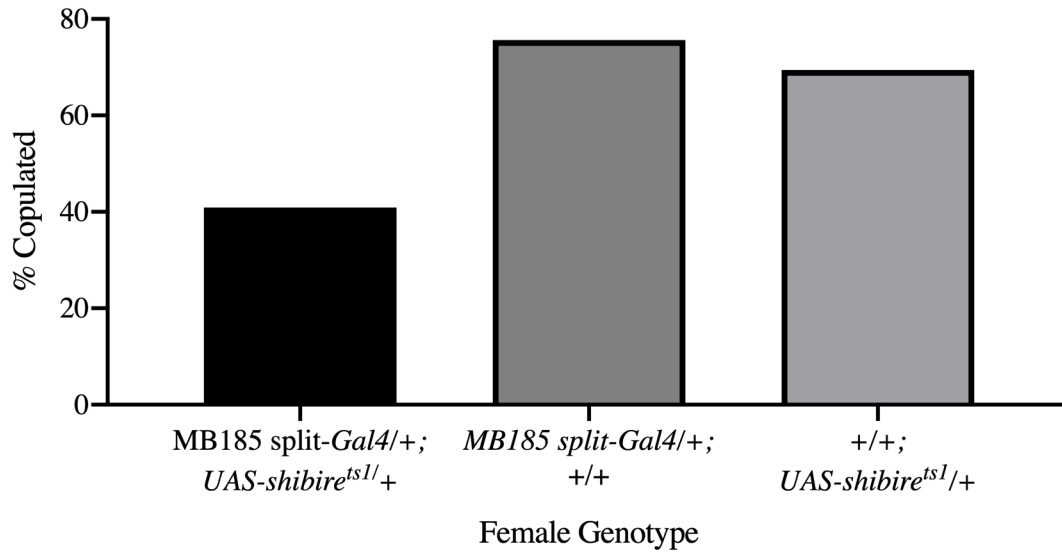


Figure 9. Silencing of the alpha / beta lobes of the MB via expression of *shibire^{ts1}* by the MB185 split-*Gal4* driver causes a reduced proportion of copulating females ($n = 44/41/36$, $\chi^2 = 12.39$, $df = 2$, $p = 0.002$, pairwise chi vs. split-*Gal4* control $p = 0.001$, pairwise chi vs. *UAS* control $p = 0.01$). Bars represent the percentage of copulated females of those that were courted.

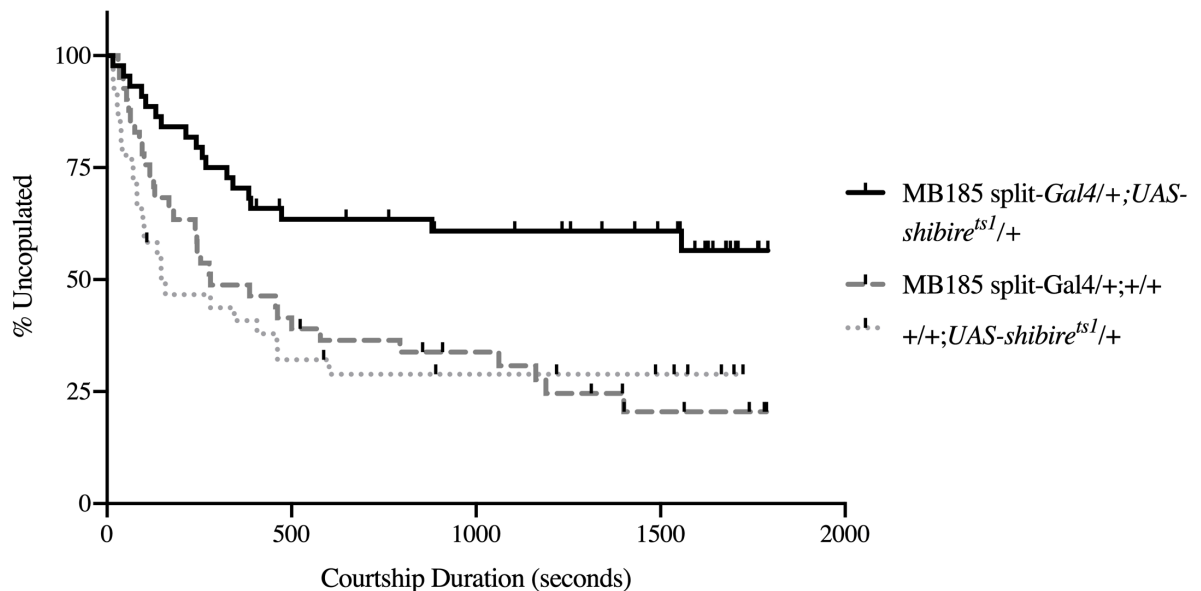


Figure 10. Silencing of the alpha / beta lobes of the MB via expression of *shibire^{ts1}* by the MB185 split-*Gal4* driver causes prolonged courtship duration ($n = 44/41/36$, Kaplan Meier concordance = 0.633, Logrank test $p = 0.002$, pairwise logrank vs. *Gal4* control $p = 0.0026$, pairwise logrank vs. *UAS* control $p = 0.0026$). Curve represents proportion of copulated females over time. Tick marks denote Kaplan Meier censorship – assay ended before copulation occurred.

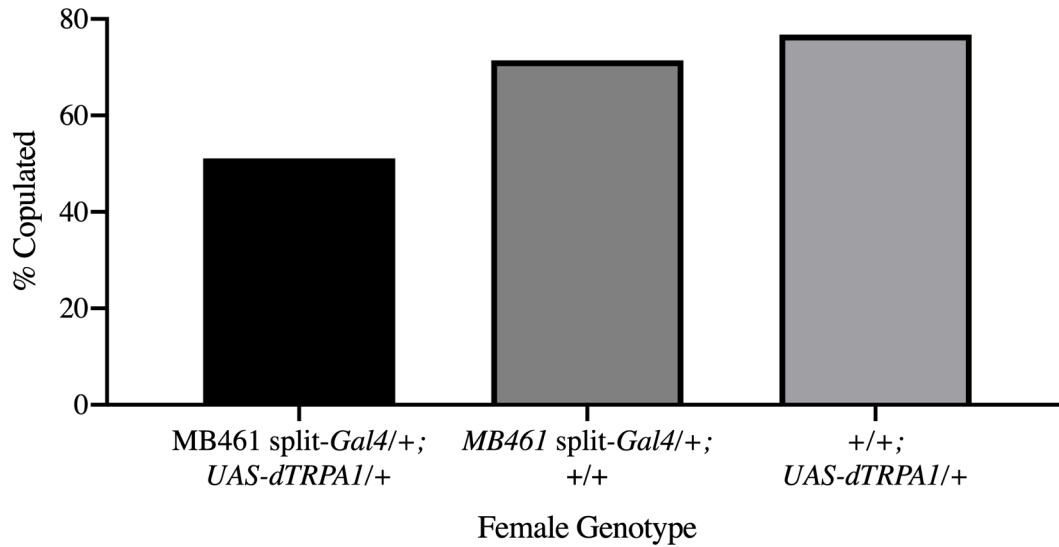


Figure 11. Hyperactivation of the alpha prime / beta prime lobes MB via expression of *dTRPA1* by the MB461 split-*Gal4* driver causes a reduced proportion of copulating females ($n = 45/42/43$, $\chi^2 = 7.22$, $df = 2$, $p = 0.027$, not significant following FDR correction, pairwise chi vs. split-*Gal4* control $p = 0.052$, pairwise chi vs. *UAS* control $p = 0.012$). Bars represent the percentage of copulated females of those that were courted.

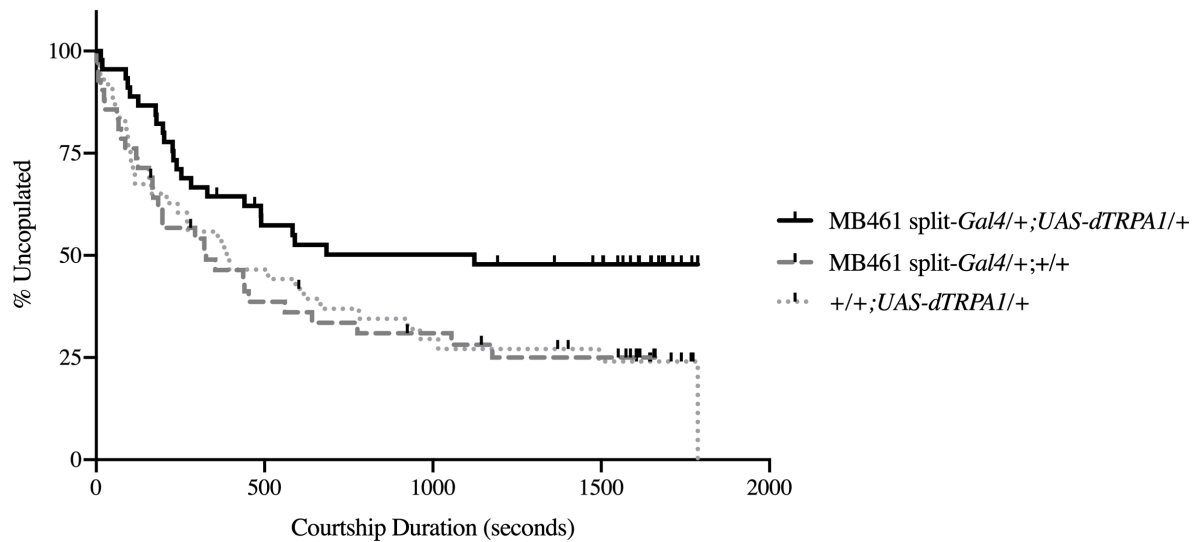


Figure 12. Hyperactivation of the alpha prime / beta prime lobes of the MB via expression of *dTRPA1* by the MB461 split-*Gal4* driver causes prolonged courtship duration ($n = 45/42/43$, Kaplan Meier concordance = 0.573, Logrank test $p = 0.03$, not significant after FDR correction, pairwise logrank vs. *Gal4* control $p = 0.037$, pairwise logrank vs. *UAS* control $p = 0.037$). Curve represents proportion of copulated females over time. Tick marks denote Kaplan Meier censorship – assay ended before copulation occurred.

Table 2. Summary of mushroom body specific silencing on female receptivity.

Anatomical Expression	Driver	Genotype	# Assayed/ # Courted	% copulated of courted	Chi Square p	Chi Square Value	Pairwise x ² p treatment vs. <i>Gal4</i> control	Pairwise x ² p treatment vs. <i>UAS</i> control	Kaplan Meier Logrank Statistic	Logrank P	Pairwise Logrank p treatment vs. <i>Gal4</i> Control	Pairwise Logrank p treatment vs. <i>UAS</i> Control
Whole MB	<i>rutabaga</i>	<i>Gal4/UAS</i>	81/46	24.44%								
		<i>Gal4/wt</i>	81/36	50.00%	0.0232	7.525	0.0171	0.0171	6.26	0.04	0.04	0.04
		<i>wt/UAS</i>	81/36	50.00%								
	MB152	<i>Gal4/UAS</i>	49/32	31.25%								
		<i>Gal4/wt</i>	49/29	65.52%	0.0129	8.6981	0.0074	0.0199	8.66	0.01	0.011	0.067
		<i>wt/UAS</i>	49/24	62.50%								
Alpha Prime / Beta Prime	MB005	<i>Gal4/UAS</i>	61/25	60.00%								
		<i>Gal4/wt</i>	61/29	37.93%	0.0049	10.626	0.1056	0.1343	11.33	0.003	0.0031	0.1231
		<i>wt/UAS</i>	61/36	77.78%								
	MB461	<i>Gal4/UAS</i>	52/26	42.31%								
		<i>Gal4/wt</i>	52/18	50.00%	0.0187	7.9636	0.6144	0.0055	16.92	0.0004	0.2905	0.0002
		<i>wt/UAS</i>	52/15	86.67%								
Alpha / Beta	MB008	<i>Gal4/UAS</i>	70/35	37.14%								
		<i>Gal4/wt</i>	70/34	50.00%	0.4735	1.4953	0.2814	0.9759	0.59	0.7	0	0
		<i>wt/UAS</i>	70/32	37.50%								
	MB185	<i>Gal4/UAS</i>	63/44	40.91%								
		<i>Gal4/wt</i>	63/41	75.61%	0.0022	12.24	0.0012	0.0109	12.65	0.002	0.0026	0.0026
		<i>wt/UAS</i>	63/36	69.44%								
Gamma	MB009	<i>Gal4/UAS</i>	51/28	53.57%								
		<i>Gal4/wt</i>	51/32	59.38%	0.8897	0.2336	0.6508	0.9453	0.29	0.9	0	0
		<i>wt/UAS</i>	51/22	54.55%								
	MB131	<i>Gal4/UAS</i>	47/22	18.18%								
		<i>Gal4/wt</i>	47/27	37.04%	0.103	4.5464	0.1462	0.0329	4.43	0.1	0	0
		<i>wt/UAS</i>	47/30	46.67%								
Gamma Subregion	MB419	<i>Gal4/UAS</i>	46/21	66.67%								
		<i>Gal4/wt</i>	46/14	71.43%	0.9567	0.0886	0.7662	0.8933	1.15	0.6	0	0
		<i>wt/UAS</i>	46/16	68.75%								
	MB607	<i>Gal4/UAS</i>	56/26	46.15%								
		<i>Gal4/wt</i>	56/37	59.46%	0.2372	2.878	0.2969	0.0922	3.8	0.1	0	0
		<i>wt/UAS</i>	56/26	69.23%								

Table 3. Summary of mushroom body specific hyperactivation on female receptivity.

Anatomical Expression	Driver	Genotype	# Assayed/ # Courted	% copulated of courted	Chi Square p	Chi Square Value	Pairwise x ² p treatment vs. <i>Gal4</i> control	Pairwise x ² p treatment vs. <i>UAS</i> control	Kaplan Meier Logrank Statistic	Logrank P	Pairwise Logrank p treatment vs. <i>Gal4</i> Control	Pairwise Logrank p treatment vs. <i>UAS</i> Control
Whole MB	<i>rutabaga</i>	<i>Gal4/UAS</i>	65/38	71.05%	0.266153	2.647	0.113945	0.697499	1.79	0.4	0	0
		<i>Gal4/wt</i>	65/27	51.85%								
		<i>wt/UAS</i>	65/30	66.67%								
	MB152	<i>Gal4/UAS</i>	45/30	70.00%	0.542212	1.224	0.337744	0.331789	0.63	0.7	0	0
		<i>Gal4/wt</i>	45/26	57.69%								
		<i>wt/UAS</i>	45/31	58.06%								
Alpha Prime / Beta Prime	MB005	<i>Gal4/UAS</i>	46/25	68.00%	0.106333	4.482	0.042254	0.751	1.49	0.5	0	0
		<i>Gal4/wt</i>	46/30	90.00%								
		<i>wt/UAS</i>	46/32	71.88%								
	MB461	<i>Gal4/UAS</i>	64/45	51.11%	0.027003	7.223	0.052282	0.012465	6.79	0.03	0.037	0.037
		<i>Gal4/wt</i>	64/42	71.43%								
		<i>wt/UAS</i>	64/43	76.74%								
Alpha / Beta	MB008	<i>Gal4/UAS</i>	51/25	48.00%	0.298132	2.420	0.162275	0.194275	2.5	0.3	0	0
		<i>Gal4/wt</i>	51/30	66.67%								
		<i>wt/UAS</i>	51/29	65.52%								
	MB185	<i>Gal4/UAS</i>	63/45	68.89%	0.953033	0.096	0.764164	0.835406	0.19	0.9	0	0
		<i>Gal4/wt</i>	63/41	65.85%								
		<i>wt/UAS</i>	63/33	66.67%								
Gamma	MB009	<i>Gal4/UAS</i>	44/27	66.67%	0.272062	2.603	0.640229	0.111181	1.61	0.4	0	0
		<i>Gal4/wt</i>	44/29	72.41%								
		<i>wt/UAS</i>	44/32	84.38%								
	MB131	<i>Gal4/UAS</i>	52/34	38.24%	0.010389	9.133	0.287561	0.002633	13.12	0.001	0.2956	0.0015
		<i>Gal4/wt</i>	52/27	51.85%								
		<i>wt/UAS</i>	52/32	75.00%								
Gamma Subregion	MB419	<i>Gal4/UAS</i>	58/40	60.00%	0.909232	0.190	0.707184	0.707184	0.86	0.7	0	0
		<i>Gal4/wt</i>	58/39	64.10%								
		<i>wt/UAS</i>	58/39	64.10%								
	MB607	<i>Gal4/UAS</i>	63/51	56.86%	0.474035	1.492	0.300561	0.880418	1.24	0.5	0	0
		<i>Gal4/wt</i>	63/40	67.50%								
		<i>wt/UAS</i>	63/38	55.26%								

3.3 SIFamide

The identification of the MBs as a modulator of female receptivity warrants further experiments to delineate the specific neural circuitry through which the MB elicits these effects. As mentioned previously, the neuropeptide SIFamide has also been implicated in female receptivity (Terhzaz et al. 2007). Experiments were performed to determine if SIFamide conveys its effects on female receptivity through action on the MBs.

3.3.1 SIFamide receptor knockdown in the MB does not influence female receptivity.

Expression of the SIFamide receptor RNAi in the whole MB or the gamma lobe did not result in any notable change to female receptivity (Table 4). Comparison of proportion of copulating females in *rutabaga-Gal4/+;UAS-SIFamideRRNAi/+* vs. genetic controls produced a significant result ($\chi^2 = 6.78$, $df = 2$, $p = 0.03$), however pairwise comparisons revealed a control genotype was the major contributor to the identified differences. The same is true for courtship duration (Kaplan Meier Logrank $p = 0.02$).

3.4 40F04 Aggression

Female receptivity or rejection are only two of many potential behavioural responses females can display toward other flies. Another example of a potential response, arguably in theme with female rejection, is aggression. This section explores the effects of hyperactivating a subset of *dsx* neurons on aggressive behaviour.

3.4.1 Hyper-activation of 40F04 neurons induces aggression and excessive grooming in females

40F04-*Gal4/+;UAS-dTRPA1/+* females were observed displaying high amounts of several different types of aggressive behaviour when paired with wildtype *D. melanogaster* males (Figure 13). The almost complete lack of aggressive behaviour in control genotype females, resulting in mostly zero values, prevented the use of most conventional statistical tests. As

opposed to comparing the precise quantities of aggressive behaviour between genotypes, the Kruskal Wallis test was employed to rank flies from least to most aggressive and subsequently test the distribution of female genotypes over the ranking. Not surprisingly, this test showed hugely significant differences in total aggressions between groups (Kruskal-Wallis $\chi^2 = 26.681$, $df = 2$, $p = 4.73 \times 10^{-6}$). The Dunn post hoc test revealed significant differences between 40F04-*Gal4/+;UAS-dTRPA1/+* females and both controls, but no difference between controls (pairwise Kruskal-Wallis vs. *Gal4* control $p = 5.06 \times 10^{-5}$, pairwise Kruskal-Wallis vs. *UAS* control $p = 5.06 \times 10^{-5}$).

40F04-*Gal4/+;UAS-dTRPA1/+* females also displayed a second phenotype – frequent head grooming (Figure 13). Head grooming instances were found to be statistically significant between groups (Kruskal-Wallis $\chi^2 = 21.137$, $df = 2$, $p = 2.57 \times 10^{-5}$), with post hoc analysis confirming differences between 40F04-*Gal4/+;UAS-dTRPA1/+* females and both controls, and no differences between controls (pairwise Kruskal-Wallis vs. *Gal4* control $p = 1.08 \times 10^{-2}$, pairwise Kruskal-Wallis vs. *UAS* control $p = 1.54 \times 10^{-5}$). This grooming phenotype from hyperactivating these particular neurons has been corroborated by literature (Seeds et al. 2014).

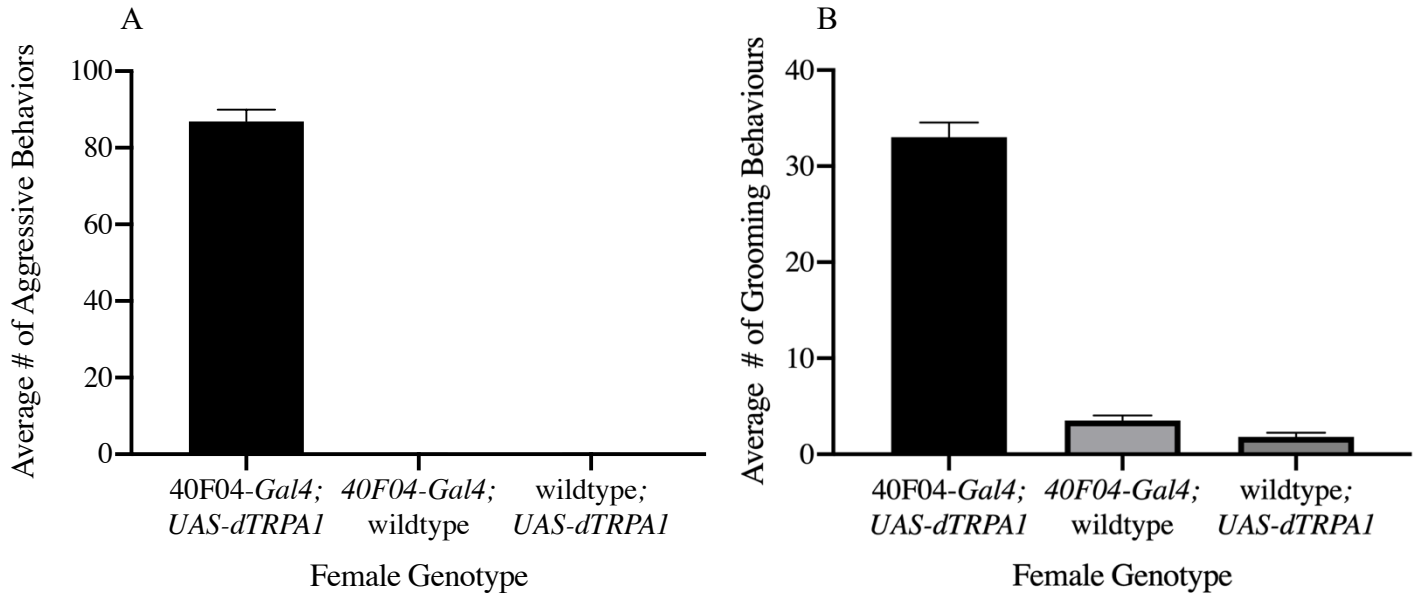


Figure 13. Hyperactivation of *dsx* pC1 & pC2 neurons defined by the 40F04-*Gal4* (A) incites high levels of female aggression toward wildtype *D. melanogaster* males ($n = 10$, Kruskal-Wallis $\chi^2 = 26.681$, $df = 2$, $p = 4.73 \times 10^{-6}$, pairwise Dunn test vs. *Gal4* control $p = 5.06 \times 10^{-5}$, pairwise Dunn test vs. *UAS* control $p = 5.06 \times 10^{-5}$) and (B) incites high levels of head grooming ($n = 10$, Kruskal-Wallis $\chi^2 = 21.137$, $df = 2$, $p = 2.57 \times 10^{-5}$, pairwise Dunn test vs. *Gal4* control $p = 1.08 \times 10^{-2}$, pairwise Dunn test vs. *UAS* control $p = 1.54 \times 10^{-5}$).

3.4.2 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females display aggression toward heterospecific males and conspecific females

When paired with wildtype *Drosophila simulans* males, 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females continue to display aggressive and grooming behaviors, while control genotype females do not (Figure 14). Kruskal Wallis analysis showed that a statically significant difference in total aggression exists between experimental groups (Kruskal-Wallis $\chi^2 = 25.003$, $df = 2$, $p = 3.72 \times 10^{-6}$). Dunn post hoc analysis confirmed the difference identified by Kruskal Wallis test is the result of 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females and not differences between controls (pairwise Dunn test vs. *Gal4* control $p = 1.78 \times 10^{-4}$, pairwise Dunn test vs. *UAS* control $p = 1.33 \times 10^{-5}$). Much of the same is true for the persistence of the head grooming phenotype with this new partner. A Kruskal Wallis test showed a significant difference between female genotypes with regard to head grooming, and Bunn post hoc test

showed the differences to be localized 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females and not controls (Kruskal-Wallis $\chi^2 = 20.434$, $df = 2$, $p = 3.65 \times 10^{-5}$, pairwise Dunn test vs. *Gal4* control $p = 1.75 \times 10^{-3}$, pairwise Dunn test vs. *UAS* control $p = 6.12 \times 10^{-5}$).

40F04-*Gal4*/+;*UAS-dTRPA1*/+ females also retain aggressive and head grooming phenotypes when partnered with wildtype *Drosophila melanogaster* females (Figure 14). Once again, Kruskal Wallis tests revealed significant differences in both total aggression (Kruskal-Wallis $\chi^2 = 21.827$, $df = 2$, $p = 1.82 \times 10^{-5}$, pairwise Dunn test vs. *Gal4* control $p = 5.97 \times 10^{-5}$, pairwise Dunn test vs. *UAS* control $p = 4.65 \times 10^{-4}$) and head grooming (Kruskal-Wallis $\chi^2 = 9.58$, $df = 2$, $p = 0.008$, pairwise Dunn test vs. *Gal4* control $p = 0.005$, pairwise Dunn test vs. *UAS* control $p = 0.354$) between female genotypes.

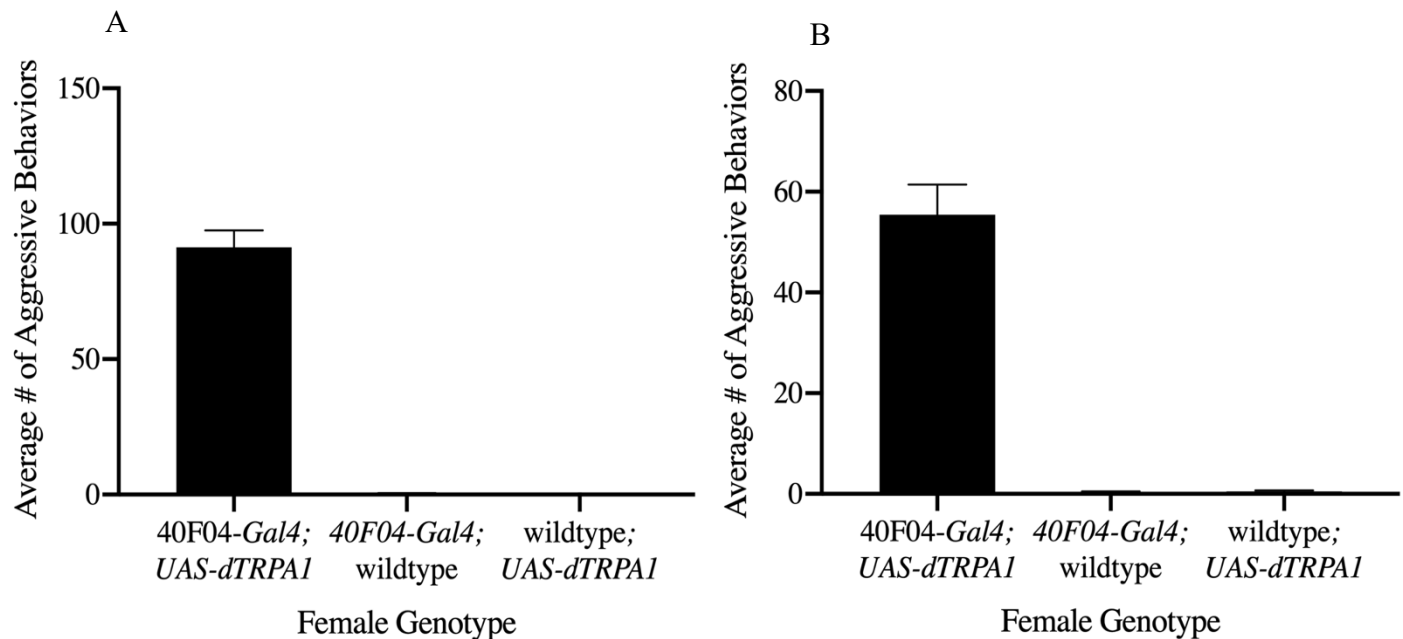


Figure 14. 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females display aggression toward (A) wildtype *D. simulans* males ($n = 10$, Kruskal-Wallis $\chi^2 = 25.003$, $df = 2$, $p = 3.72 \times 10^{-6}$, pairwise Kruskal-Wallis vs. *Gal4* control $p = 1.78 \times 10^{-4}$, pairwise Dunn test vs. *UAS* control $p = 1.33 \times 10^{-5}$), and (B) wildtype *D. melanogaster* females ($n = 10$, Kruskal-Wallis $\chi^2 = 21.827$, $df = 2$, $p = 1.82 \times 10^{-5}$, pairwise Dunn test vs. *Gal4* control $p = 5.97 \times 10^{-5}$, pairwise Dunn test vs. *UAS* control $p = 4.65 \times 10^{-4}$).

3.4.3 Partner type affects amount of specific aggressive behaviours in 40F04-*Gal4/+;UAS-dTRPA1/+* females

Although 40F04-*Gal4/+;UAS-dTRPA1/+* females display aggressive behaviours toward conspecifics of either sex as well as heterospecific males, the amounts of aggressive behaviour displayed to different partners is not equal (Figure 15). Use of a Poisson logistic regression to compare total aggression counts for 40F04-*Gal4/+;UAS-dTRPA1/+* females paired with each partner type showed a statistically significant difference exists (Poisson regression $z = 8.28$, $p = 2.0 \times 10^{-16}$). A modified Tukey post hoc test was used to assess the pair-wise differences in aggression counts for each partner type. *D. melanogaster* males and *D. simulans* males were found to not be statistically different with regard to total aggression counts, whereas *D. melanogaster* females were found to receive statistically less aggression than both *D. melanogaster* males and *D. simulans* males (post hoc pairwise Tukey contrasts *melanogaster* males vs. *simulans* males $p = 0.297$, *melanogaster* males vs. *melanogaster* females $p = 2.22 \times 10^{-16}$, *simulans* males vs. *melanogaster* females $p = 2.0 \times 10^{-16}$). This remains true for most specific aggressive behaviours (Table 5), with the exception of headbutts (shoving). Headbutts were more commonly displayed by 40F04-*Gal4/+;UAS-dTRPA1/+* females paired with *D. melanogaster* females (Poisson regression $z = -5.673$, $p = 1.4 \times 10^{-8}$, pairwise *melanogaster* female vs. *melanogaster* male Tukey $p = 2.0 \times 10^{-16}$, *melanogaster* female vs. *simulans* male Tukey $p = 1.4 \times 10^{-8}$). Additionally, head grooming counts were less frequent in 40F04-*Gal4/+;UAS-dTRPA1/+* females paired with *D. melanogaster* females (Poisson regression $z = 6.849$, $p = 7.46 \times 10^{-12}$, pairwise *melanogaster* female vs. *melanogaster* male Tukey $p = 7.46 \times 10^{-12}$, *melanogaster* female vs. *simulans* male Tukey $p = 1.24 \times 10^{-14}$).

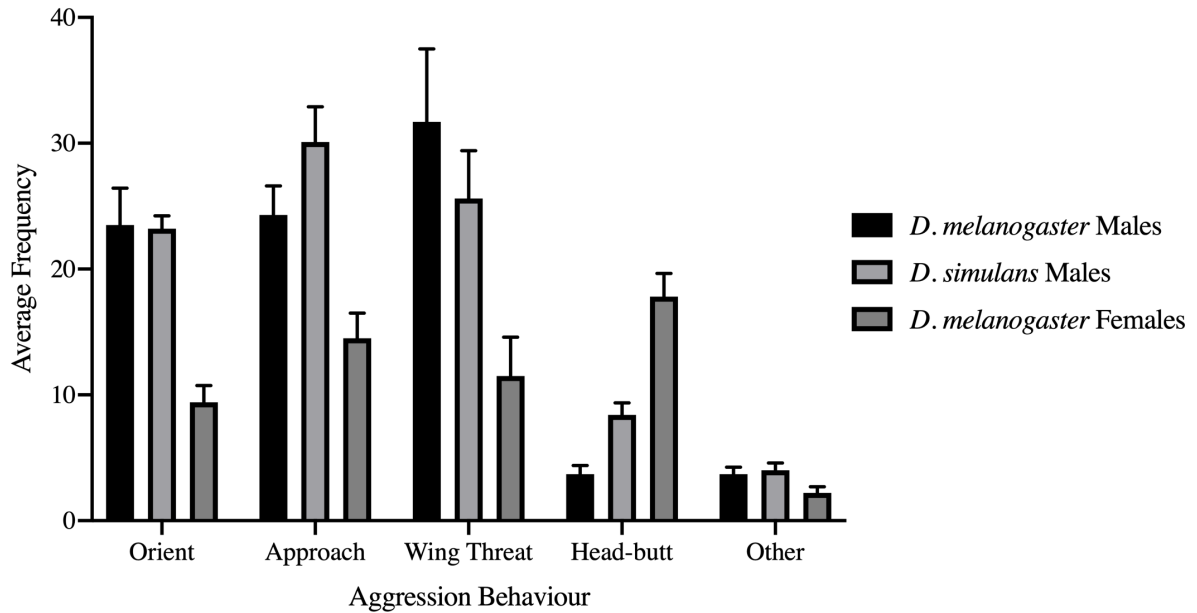


Figure 15. 40F04-*Gal4/+;UAS-dTRPA1/+* females display more aggression toward males, regardless of species ($n = 10$, Total aggression Poisson regression $z = 8.28$, $p = 2.0 \times 10^{-16}$, post hoc pairwise Tukey contrasts *melanogaster* males vs. *simulans* males $p = 0.297$, *melanogaster* males vs. *melanogaster* females $p = 2.22 \times 10^{-16}$, *simulans* males vs. *melanogaster* females $p = 2.0 \times 10^{-16}$).

Table 5. Statistical differences of aggressive behaviours displayed by 40F04-*Gal4/+;UAS-dTRPA1/+* toward *D. melanogaster* males, *D. melanogaster* females, and *D. simulans* males.

Behaviour	Poisson z value	Poisson p value	Post hoc Tukey p <i>mel</i> M vs. <i>sim</i> M	Post hoc Tukey p <i>mel</i> M vs. <i>mel</i> F	Post hoc Tukey p <i>sim</i> M vs. <i>mel</i> F
Total	8.28	$2.0 \times 10^{-16}***$	0.297	$2.0 \times 10^{-16}***$	$2.22 \times 10^{-16}***$
Orient	7.389	$1.48 \times 10^{-13}***$	0.89	$6.0 \times 10^{-14}***$	$1.48 \times 10^{-13}***$
Approach	4.92	$8.64 \times 10^{-7}***$	0.0131*	$8.64 \times 10^{-7}***$	$5.0 \times 10^{-13}***$
Wing Threat	7.129	$1.01 \times 10^{-12}***$	0.011*	$2.0 \times 10^{-16}***$	$1.01 \times 10^{-12}***$
Head-butt	-5.673	$1.4 \times 10^{-8}***$	$3.25 \times 10^{-5}***$	$2.0 \times 10^{-16}***$	$1.40 \times 10^{-8}***$
Other	1.931	0.053	0.733	0.535	0.0243*
Grooming	6.849	$7.46 \times 10^{-12}***$	0.434	$7.46 \times 10^{-12}***$	$1.24 \times 10^{-14}***$

Evaluation of whether movement is necessary to incite aggressive behaviours from 40F04-*Gal4/+;UAS-dTRPA1/+* females was conducted by pairing 40F04-*Gal4/+;UAS-dTRPA1/+* females with various partners varying in the stimuli they present. Hyper-locomotive females were used to assess if the lower amounts of aggression directed toward wildtype *melanogaster* females is potentially a product of differences in locomotion between the sexes. The amount of aggression displayed toward hyperlocomotive females was considerably higher than aggression toward wildtype *melanogaster* females (Figure 16; Poisson regression $z =$, $p = 2.0 \times 10^{-16}$, pairwise hyperlocomotive female vs. wt *melanogaster* female Tukey $p = 2.0 \times 10^{-16}$). Headless wildtype *D. melanogaster* males, which retain normal male chemical cues but lack male behaviour, were used to test whether male chemical cues or visual presence are sufficient to elicit 40F04-*Gal4/+;UAS-dTRPA1/+* female aggression. Interestingly, and potentially amusingly, 40F04-*Gal4/+;UAS-dTRPA1/+* females also display aggressive behaviours toward these headless male partners (Figures 16 & 17)(Kruskal-Wallis $\chi^2 = 21.8$, $df = 2$, $p = 1.82 \times 10^{-5}$, pairwise Kruskal-Wallis vs. *Gal4* control $p = 5.97 \times 10^{-5}$, pairwise Kruskal-Wallis vs. *UAS* control $p = 4.65 \times 10^{-4}$). However, headless males incite less aggression than live wildtype *melanogaster* males (Figure 16; Poisson regression $z =$, $p = 2.0 \times 10^{-16}$, pairwise headless male vs. wt *melanogaster* male Tukey $p = 2.0 \times 10^{-16}$). Preliminary experiments have also shown extreme aggression between a pair of 40F04-*Gal4/+;UAS-dTRPA1/+* females. Aggression between this pairing lacks formal experimentation and quantification, but observations include escalated aggression behaviours not seen in any previous partner type, including boxing and tussling.

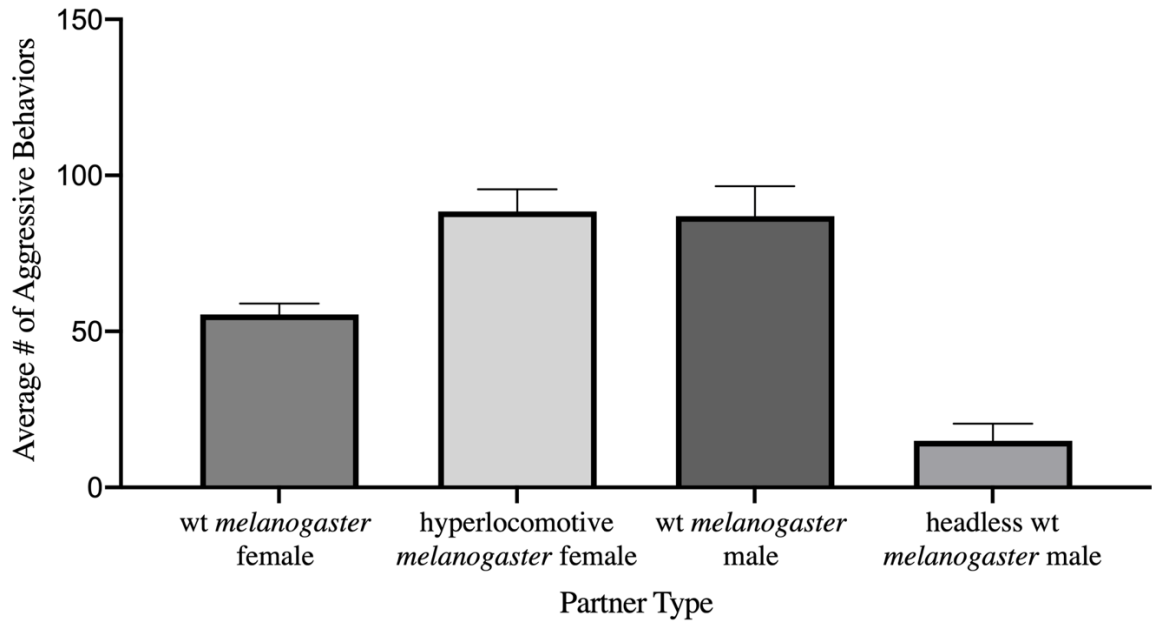


Figure 16. Hyperlocomotive females and headless males both receive aggression from 40F04-*Gal4/+;UAS-dTRPA1/+* females. Hyperlocomotive females receive more aggression than wt females ($n = 10$, Poisson regression $z =$, $p = 2.0 \times 10^{-16}$, pairwise hyperlocomotive female vs. wt *melanogaster* female Tukey $p = 2.0 \times 10^{-16}$), while headless males receive less aggression than their live wt male counterparts ($n = 10$, Poisson regression $z =$, $p = 2.0 \times 10^{-16}$, pairwise headless male vs. wt *melanogaster* male Tukey $p = 2.0 \times 10^{-16}$).

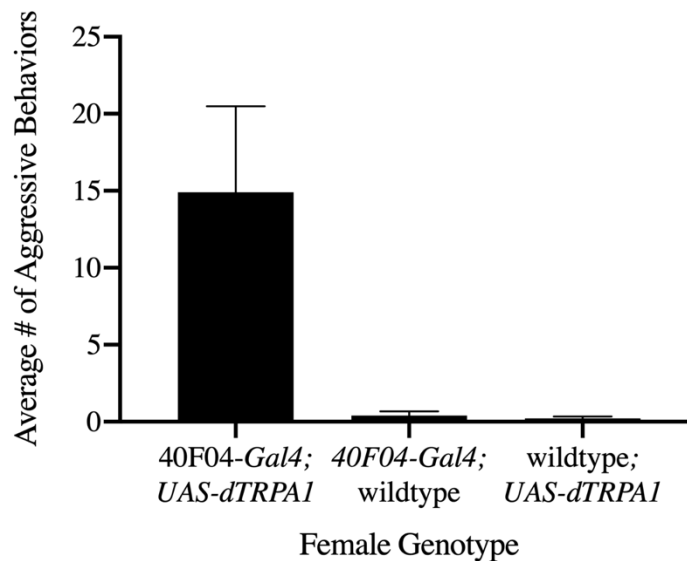


Figure 17. 40F04-*Gal4/+;UAS-dTRPA1/+* female aggression toward headless males is significantly higher than controls ($n = 10$, Poisson regression $z =$, $p = 2.0 \times 10^{-16}$, pairwise headless male vs. wt *melanogaster* male Tukey $p = 2.0 \times 10^{-16}$).

3.4.4 Preliminary: Females with split-*Gal4* targeted hyper-activation of 40F04 neurons also expressing *dsx* retain aggressive phenotypes but lack excessive grooming

Split-*Gal4* hemidriviers were used together to restrict expression of *dTRPA1* exclusively to 40F04 neurons expressing *dsx*. 71042, an AD hemidriver version of 40F04, was paired with a *dsx*DBD hemidriver as well as *UAS-dTRPA1*. Five females containing all three transgenics were observed to retain the aggressive phenotype but lacked the grooming phenotype. Qualitatively, the aggression displayed by 71042/*UAS-dTRPA1*;*dsx*DBD-split-*Gal4* females was less intense than that of binary 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females. However, these observations are preliminary and require proper controls and quantification prior to formal analysis.

3.4.5 40F04-*Gal4*/+;*UAS-dTRPA1*/+ males display grooming phenotype and substantially less aggression than females

40F04-*Gal4*/+;*UAS-dTRPA1*/+ males paired with wildtype *D. melanogaster* males were shown to exhibit the excessive grooming phenotype observed in 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females, and display a slight but significant increase in aggression (Figure 18). However, these males lack the extreme aggression observed in 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females (Figure 18; note difference in y-axis scale). 40F04-*Gal4*/+;*UAS-dTRPA1*/+ males were found to have significantly higher counts of total aggression behaviours than genetic controls (Kruskal-Wallis $\chi^2 = 11.478$, $df = 2$, $p = 0.003$). However, 40F04-*Gal4*/+;*UAS-dTRPA1*/+ male aggression is not nearly as prevalent as 40F04-*Gal4*/+;*UAS-dTRPA1*/+ female aggression – the minimum and maximum counts of total aggression from 40F04-*Gal4*/+;*UAS-dTRPA1*/+ males are 0 and 9 (mean = 2.3), while 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females range from 17 to 115 (mean = 86.9). Statistical comparison of 40F04-*Gal4*/+;*UAS-dTRPA1*/+ males and 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females paired with wildtype *D. melanogaster* males showed a significantly higher amount of aggression in 40F04-*Gal4*/+;*UAS-dTRPA1*/+ females (Kruskal-Wallis $\chi^2 = 14.5$, $df = 1$, $p = 0.0001$).

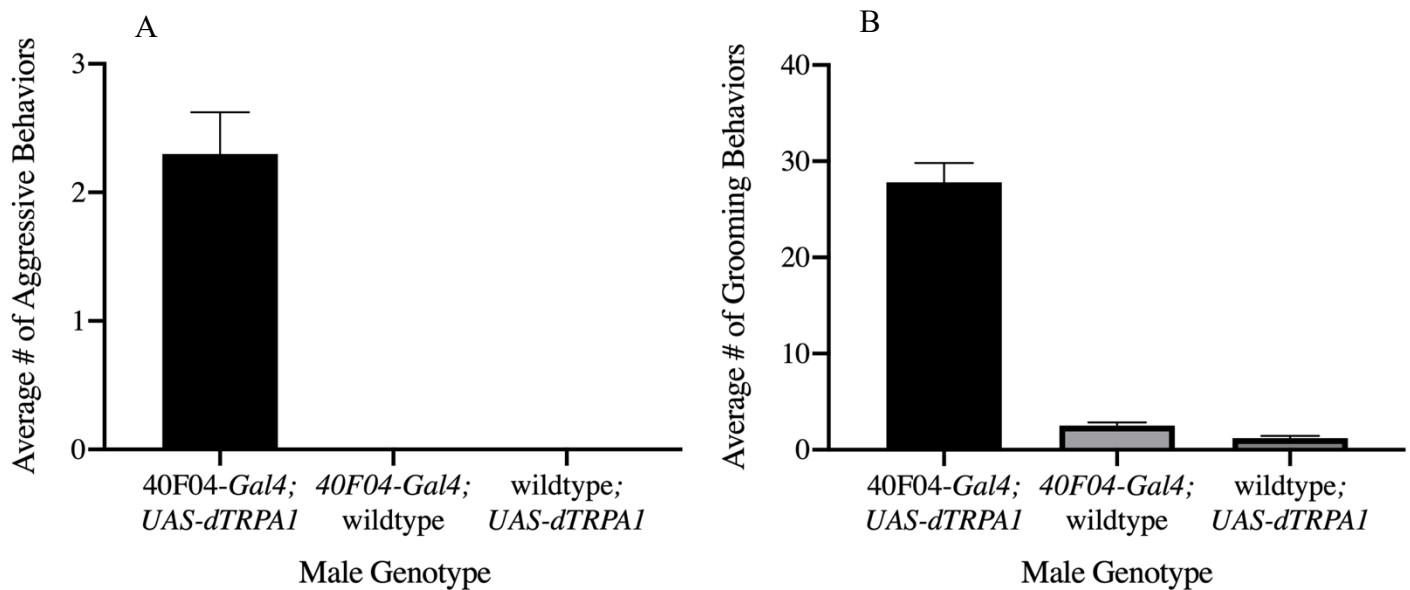


Figure 18. 40F04-*Gal4/+;UAS-dTRPA1/+* males display (A) significant but low amounts of aggression ($n = 10$, Kruskal-Wallis $\chi^2 = 11.478$, $df = 2$, $p = 0.003$; note reduced scale of y-axis compared to those used for females), and (B) excessive grooming ($n = 10$, Kruskal-Wallis $\chi^2 = 25.157$, $df = 2$, $p = 3.44 \times 10^{-6}$).

Additionally, in preliminary experiments 71042 split-*Gal4AD/wt;dsxDBD/UAS-dTRPA1* males ($n = 9$) were qualitatively observed to also lack the aggression phenotype, much like 40F04-*Gal4/+;UAS-dTRPA1/+* males. Grooming was also not observed in 71042 split-*Gal4AD/+;dsxDBD/UAS-dTRPA1* males, consistent with the observations of 71042 split-*Gal4AD/+;dsxDBD/UAS-dTRPA1* females. However, 71042 split-*Gal4AD/+;dsxDBD/UAS-dTRPA1* males were observed courting both male and female partners. Formal experimentation is needed to determine if this courtship behaviour is statistically different than control genotypes.

4 Discussion

4.1 Silencing the Mushroom Body Reduces Female Receptivity

The mushroom bodies (MB) have been proposed as an area of sensory integration (Davis 1993; Yagi et al. 2016), and thus I predicted that manipulation of this neural structure would also affect female receptivity. As hypothesized, manipulation of the MB (specifically silencing) was shown to affect female receptivity. Expression of *shibire^{ts1}* using two unique MB-specific *Gal4* driver lines, *rutabaga-Gal4* and MB152 split-*Gal4*, produced near identical differences between treatment and control genotypes. The differences observed in these experiments retained their statistical significance following a false discovery rate correction for multiple testing. This strong effect witnessed from two unique drivers expressing in the same anatomical region demonstrates the MB is biologically important to the process of female receptivity.

In contrast to the result that silencing the MB decreases receptivity, it has previously been shown that ablation of the MBs is insufficient to produce changes in female receptivity (Neckameyer 1998a). Although silencing and ablation are intuitively similar (both result in a lack of outgoing signal), the discrepancy of these results may highlight a functional difference between these means of neural activity modification, especially with regard to temporal modifications. In the Neckamayer experiment, ablation occurred early in development, meaning alternative compensatory circuitry may have developed, masking the true behavioural effects of lacking the MB. Alternatively, the elimination of these neurons early in development may have prevented formation of particular adjacent neural connections that are critical for rejection behaviour to be performed. Silencing the MB exclusively in adults during behavioural experimentation, as I did, bypasses these potential problems.

While silencing of the MB produced differences in female receptivity, hyperactivation of the same region using the same *Gal4* drivers produced no such differences in behaviour. One might expect, given that silencing the MB decreases receptivity, that hyperactivation of the MB would increase receptivity. There are several potential reasons why this may not be the case. Firstly, if the MB is indeed an area of sensory integration, it may exert its effects on

female receptivity exclusively through proper processing of courtship-relevant stimuli, and not by elicitation of behavioural responses. Silencing the MB would then prevent proper sensory processing, leading to lack of proper courtship evaluation/recognition and ultimately lack of copulation. In this hypothesis, if the MB were to be hyper-activated, the only result would be more “active” processing of sensory information, although the evaluation of courtship quality and subsequent response would be unchanged. This would explain the lack of difference female receptivity between females with hyperactivated MBs when compared to their genetic controls. An alternative explanation is that a brain region downstream of the MB requires sufficient MB signaling to elicit acceptance behaviours. The lack of such signaling as seen in MB-silenced females results in slower and less frequent acceptance. A surplus of such signaling as seen in MB-hyperactivated females does not induce more acceptance, potentially through saturation of the signaling required by the downstream region to elicit acceptance. Both of these interpretations, however, are somewhat at odds with the previously discussed study showing the ablation of MBs is insufficient to cause differences in female receptivity (Neckameyer 1998a). Given the structural and functional complexity of the MB, is also possible that the MB is involved in several processes relevant to female receptivity and that its hyperactivation causes opposing behavioural changes elicited by different sub regions to mask one another. For this last reason, it is essential to assess the roles of specific regions within the MB.

4.2 Individual Lobes of the Mushroom Body Influence Female Receptivity

Silencing, but not hyperactivating, the alpha / beta lobes was found to significantly affect female receptivity. These lobes were assessed using two different lobe-specific drivers: MB008 and MB185. Interestingly, *shibire^{ts1}* expressed by the MB008 split-*Gal4*, a driver also specific to the alpha / beta lobes, did not replicate the significant effect seen in MB185 split-*Gal4*; *UAS-shibire^{ts1}* females. This highlights a major motivation for using multiple drivers specific to the same anatomical region: *Gal4* and split-*Gal4* drivers are not perfect representations of anatomy. For example, a *Gal4* driver may express exclusively within the alpha / beta lobes, but that does not mean *all* alpha / beta cells are being manipulated. Though they both express within the alpha / beta lobes, MB185 and MB008 are different

drivers composed of different enhancer regions. This means that the specific cells expressing *Gal4* would be expected to be slightly different between MB185 and MB008. MB185 was sufficient to reveal an effect, but MB008 may have been ineffective in manipulating the precise neurons required to reveal the influence of the alpha / beta lobes on female receptivity.

Hyperactivation of the alpha / beta lobes in MB185 split-*Gal4;UAS-dTRPA1* females did not produce changes in female receptivity. As silencing these neurons produced a robust decrease in receptivity, one might expect hyperactivation of the same neurons to cause the opposite effect of increasing receptivity. This logic was discussed previously in regard to whole-MB silencing and hyperactivation, and many of the interpretations listed there are also applicable here.

Hyperactivation of the alpha prime / beta prime lobes was also shown to decrease female receptivity for one of the lobe-specific drivers used (MB461), but the data were found not statistically significant after false discovery rate correction for multiple testing. Additionally, the other driver used to express *dTRPA1* in these lobes did not produce a statistically significant difference in female receptivity when compared to controls. Therefore, the observation of decreased receptivity when hyperactivating the alpha prime / beta prime lobes lacks 1) the corroboration by use of multiple drivers as seen in the whole MB data, and 2) robust statistical significance seen in MB185 split-*Gal4;UAS-shibire^{ts1}* females. Together, these shortcomings may be interpreted as a lack of genuine influence, or could be a false negative result, thus further investigation may be warranted.

In attempting to further delineate the roles of the alpha / beta lobes in female receptivity, it may be worthwhile to assess which neurotransmitters are necessary to produce the changes in receptivity observed here. One neurotransmitter occasionally discussed in regard to the MB and female receptivity is dopamine (Neckameyer 1998b,a; Zhang et al. 2007; Aso et al. 2014). The alpha lobes (as well as alpha prime lobes) have been shown be innervated by dopaminergic neurons, as well as express dopamine themselves (Mao 2009).

The results gathered through use of the lobe-specific drivers show that individual lobes can have an effect on female receptivity. This observation may lend credence to the idea that the anatomical and functional complexity of the MB allows for specific functions to be localized to specific areas of the structure. To ensure that this is in fact the case, the influence of other regions of the MB on female receptivity must be ruled out. Many of the data in this thesis lay the foundation for this exclusion of some MB regions as candidates for effectors of female receptivity.

4.3 The Potential Influence of Other Tested Lobes

The notion that *Gal4* and split-*Gal4* drivers are not perfect anatomical representations of their targeted brain regions, and thus may not always be capable of revealing a region's influence on a process, has broader implications for the data of this thesis. Many of the chosen drivers did not produce any significant changes in female receptivity behaviour when combined with *UAS-dTRPA1* or *UAS-shibire^{ts1}*. However, this cannot be interpreted as the targeted brain areas being unimportant for the process of female receptivity. It is entirely possible that the tested brain regions may influence female receptivity, but the chosen drivers did not reveal their effects. This could be due to conflicting effects within a single lobe, or due to the chosen drivers not being expressed in every cell of the target lobe. It may also be possible that tested lobes have subtle effects on receptivity, and manipulating them individually elicits only minor changes not deemed significant by statistical analysis. For these reasons, no broad conclusions about functional segregation within the MB can yet be made. The alpha / beta lobes can be said to influence female receptivity with a good degree of confidence, and the alpha prime beta prime lobes may also contribute.

4.4 SIFamide Receptor Knockdown in the Mushroom Body Does Not Influence Female Receptivity

Site-specific knockdown of the SIFamide receptor in both the whole mushroom body and gamma lobe of the mushroom body did not significantly affect female receptivity. The lack of difference between experimental and control genotypes seen here can be interpreted in several ways. Firstly, it is possible that the RNAi knockdown is not sufficient to completely

eradicate the receptor. There are no previously reported tests of the efficiency of the knockdown for this line, and therefore assessment confirming RNAi knockdown is needed. Alternatively, SIFamide may elicit its effects on female receptivity through action on different anatomical regions. As mentioned previously, SIFamide neurons, and the brain region they occupy, innervate various regions of the protocerebrum. Many of these regions are currently being assessed, though data will not be complete in time for inclusion in this thesis. It may also be possible that SIFamide acts on the MB and multiple other regions to effect female receptivity, and thus knocking down its receptor in only the MB is insufficient to cause the phenotypes seen in individuals with ablated SIFamide neurons – lack of SIFamide signaling in the MB may be compensated for by the other regions in the circuit.

4.5 Characterizing 40F04-*Gal4/+;UAS-dTRPA1/+* Female Behavioural Phenotype

In 2016, the Goodwin lab published a study in which they found that 40F04-*Gal4/+;UAS-dTRPA1/+* males had increased courtship, then tested this same driver in females with the goal to show that these same neurons incite courtship in females. They found that females did indeed show approach and wing behaviours, which they interpreted as male-like courtship. However, there are a few distinct differences in these behavioural patterns that ensure the observed phenotype in 40F04-*Gal4/+;UAS-dTRPA1/+* females is indeed aggression, and not male courtship. The most pronounced differences are the wing behaviours and approaches that are distinct between aggression and male courtship. The wing displays of aggressive females include wing threats and wing claps. Wing claps involve one wing being extended to a roughly 90 degree angle from the body, then returning to original position, similar to how a male holds his wing during courtship wing song (Greenspan and Ferveur 2002; Nilsen et al. 2004). Wing threats, however, involve the extension of both wings to roughly 45 degrees, a behaviour that is distinct from what is observed during male courtship displays.

Likewise, while a male will approach and gently tap a female with his foreleg during courtship, the aggressive females display high incidences of head-butting or shoving, in which the 40F04-*Gal4/+;UAS-dTRPA1/+* female will strike the partner fly with their head such that the partner fly is moved. Instances of head-butting are in part facilitated by the

partner fly, as aggressive individuals require the partner to be relatively stationary in order to elicit a head-butt or shove. Therefore, instances of head-butting and shoving are much more visually apparent when 40F04-*Gal4/+;UAS-dTRPA1/+* females are paired with more stagnant partners (wildtype *melanogaster* females or headless wildtype males). Additionally, pairing 40F04-*Gal4/+;UAS-dTRPA1/+* females together produces interactions exceptionally distinct from courtship. These flies display a number of aggressive behaviours not displayed by 40F04-*Gal4/+;UAS-dTRPA1/+* females with any other partner yet attempted. These behaviours include boxing and tussling.

4.6 40F04-*Gal4/+;UAS-dTRPA1/+* Induces Sex-Specific High Levels of Aggression

Perhaps the most exciting aspect of the female aggression phenotype I observed is the differences observed between the sexes: 40F04-*Gal4/+;UAS-dTRPA1/+* males display similar grooming to that observed in 40F04-*Gal4/+;UAS-dTRPA1/+* females, but do not display aggression nearly to the same extent. Further, the aggression that is induced in females is female-like rather than male-like in terms of the types and amounts of aggressive behaviours. Therefore, the aggression induced in 40F04-*Gal4/+;UAS-dTRPA1/+* females is sex-specific. To my knowledge, this is the first identified neural subset implicated in female-specific aggression.

Many of the aggressive behaviours demonstrated by 40F04-*Gal4/+;UAS-dTRPA1/+* females are typical of both male and female aggression, namely orienting, approaching, and wing threats. However, high frequencies of head-butting (a form of shoving) were witnessed in 40F04-*Gal4/+;UAS-dTRPA1/+* females, which is a female-specific behaviour (Nilsen et al. 2004). The presence of this behaviour in combination with the relatively high frequency at which it is observed indicates that the aggression demonstrated by 40F04-*Gal4/+;UAS-dTRPA1/+* females is stereotypically female.

It is worth consideration that previous characterization of aggression differences between the sexes has come from experiments carried out almost exclusively with same sex pairs (Nilsen et al. 2004). This was a necessary condition to prevent confounding courtship behaviours,

however it does place limits on the robustness of the produced behavioural ethograms. As was demonstrated in this thesis, types and amounts of specific aggression behaviour may vary depending on stimuli presented by the partner fly. Had females in this earlier study been made to be aggressive and instead paired with a non-courting male, they may have demonstrated different frequencies or sequences of behaviours, or perhaps even different behaviours, thus producing a different ethogram. Additionally, these ethograms are context specific – flies are made to fight over a limited resource (males over a headless female, and females over yeast paste). As flies are attempting to defend this resource, and thus positioning themselves close to it, aggression ethograms would likely be different in the absence of any resource to defend. All of these reservations are best exemplified by the pairing of 40F04-*Gal4/+;UAS-dTRPA1/+* aggressive females. 40F04-*Gal4/+;UAS-dTRPA1/+* females in this new context demonstrate different behaviours, such as boxing and tussling which were thought to be exclusively male behaviours. These ethograms are therefore more accurately representative of male vs. male and female vs. female resource competition aggression, as opposed to male and female aggression in all contexts.

Taking this into consideration, it is difficult to conclude whether there is a stereotypically sex-specific nature to the behaviours demonstrated by 40F04-*Gal4/+;UAS-dTRPA1/+* females. Although head butting is a strong indication of female-specific aggression based on the existing ethograms, males may potentially be made to demonstrate this behaviour in yet unexplored circumstances (ex. fights with less mobile partners).

With the benefit of hindsight, the role of *dsx* neurons in female aggression may have been predictable based on the observation that some *dsx* neurons have already been implicated in male aggression (Koganezawa et al. 2016). These same neurons were also shown to effect male courtship, and were proposed as a switch between these two distinct behaviours. Due to the role of *dsx* in coordinating development of sex-specific neural circuitry and its roles in female sexual behaviours, the findings here demonstrating its roles in female aggression are consistent with what might be expected based on the literature. However, there was no evidence pointing toward 40F04 neurons specifically as potential influencers of aggression. In fact, the neural switch between male aggression and courtship proposed by Koganezawa et al. occupies the pC1 cluster of *dsx* neurons, whereas the 40F04-*Gal4* expresses mainly in the

pC2 cluster (Zhou et al. 2014). pC2 is also the site of most variation when comparing 40F04-*Gal4* expression patterns between males and females, meaning it is the most likely site for neurons controlling female-specific aggression. The potential distinction of pC2 being an important site for female-specific aggression, while pC1 is important for male aggression, may be another example of *dsx* defining sex-specific neural circuitry pertinent to sex-specific behaviour. The differences in neural circuitry controlling aggressive behaviours may reflect the different situations in which males and females display aggression – for example, pC2 may hypothetically receive signals regarding copulation and might therefore be involved in post-mating female aggression.

Also interesting is the fact that the dramatic and robust aggression and grooming phenotypes are the result of a manipulation of a small subset of neurons. Furthermore, results obtained through restriction of *dTRPA1* expression to 40F04 neurons expressing *dsx* demonstrate that each phenotype is induced through manipulation of a subset of only 9 neurons (18 bilaterally)(Zhou et al. 2014).

Experiments are currently underway that serve to delineate which neurotransmitters are used by the aggression inciting 40F04 *dsx* neurons. The neurotransmitters currently under investigation are dopamine, which has previously been implicated in *Drosophila* aggression (Alekseyenko et al. 2013; Kayser et al. 2015), as well as serotonin (Johnson et al. 2009; Alekseyenko et al. 2014). Other neurotransmitters occasionally discussed in respect to aggression octopamine (Zhou et al. 2008; Andrews et al. 2014; Watanabe et al. 2017) and GABA (Alekseyenko et al. 2019). These neurotransmitters have been omitted from the assessment of 40F04 neuron aggression due to the fact that *dsx* is not co-expressed with either octopamine or GABA within the female brain (Rezával et al. 2014b; Zhou et al. 2014).

The study that developed the 40F04 *Gal4* driver found that hyperactivation of these neurons produced no significant changes in female receptivity (Zhou et al. 2014). The same conclusion was replicated by Janelia Fly Bowl tracking software (Simon and Dickinson 2010). This is seemingly at odds with the observations of high amounts of female aggression being observed in these females. Persistent female aggression should intuitively result in a

decrease in receptivity, and at bare minimum and increase in courtship duration. Perhaps, given long enough assays allowing females to exhaust themselves after several aggressive displays, partner males are capable of copulating. Grooming attempts that appear to be mutually exclusive to aggressive behaviours may also provide a window of opportunity for males to court and copulate. However, behavioural quantification of longer assays is required to fully understand this discrepancy.

4.7 40F04-*Gal4/+;UAS-dTRPA1/+* Female Aggression Inciting Stimuli

Pairing 40F04-*Gal4/+;UAS-dTRPA1/+* females with *D. melanogaster* females as well as *D. simulans* males demonstrated the induced aggression is not exclusively in response to either sex-specific or species-specific cues. This rules out courtship, species-specific courtship behaviours, or species-specific chemical cues as sole stimuli inciting 40F04-*Gal4/+;UAS-dTRPA1/+* female aggression. However, the observation that males of either species regularly receive higher counts of aggressive displays implies differences in the stimuli they present, and these differences incite more aggression from 40F04-*Gal4/+;UAS-dTRPA1/+* females.

The observation that hyper-locomotive females receive more aggression than wildtype females confirms movement as a major contributing stimulus for inciting 40F04-*Gal4/+;UAS-dTRPA1/+* female aggression. Combining these aggression data with formal quantifications of partner movement would better inform the relationship between these behaviours. Average number of aggressive behaviours directed toward hyper-locomotive females is quite similar to those seen in wildtype males (hyper-locomotive female mean = 88.4, wildtype male mean = 86.6), though quantification is required to ensure similar locomotion from these partners. Hypothetically, it may be possible that hyper-locomotive females are more mobile than wildtype males and yet receive similar amounts of aggression. This would imply the influence of other sensory modalities influencing 40F04-*Gal4/+;UAS-dTRPA1/+* female aggression. However, it is already clear that movement is not the only stimuli sufficient to elicit 40F04-*Gal4/+;UAS-dTRPA1/+* female aggression. The finding that 40F04-*Gal4/+;UAS-dTRPA1/+* females direct some amount of aggressive behaviours toward

wildtype headless males implies some role of chemical cues in eliciting 40F04-Gal4/+;UAS-dTRPA1/+female aggression. As these partner flies do not display behaviours to incite aggression, chemical cues and the mere presence of the fly are the only stimuli presented. Interestingly, chemical cues were found to influence the behaviours of 40F04-Gal4/+;UAS-dTRPA1/+females described by Rezával et al. 2016. To ensure 40F04-Gal4/+;UAS-dTRPA1/+females are not simply aggressive toward any vaguely fly-shaped object within their immediate vicinity, a fly surrogate object (such as a small piece of clay) may be used in future partner experiments. Headless oenocyteless flies lacking both behaviour and pheromones (Billeter et al. 2009) would also be useful in delineating the effects of chemical cues on 40F04-Gal4/+;UAS-dTRPA1/+female aggression. If chemical cues are a contributing factor for 40F04-Gal4/+;UAS-dTRPA1/+female aggression, use of headless females as partners may also help determine if male sex-specific chemical cues are sufficient to elicit aggression.

It is also worth noting that higher counts of aggressive behaviours does not necessarily equate to more intense aggression overall. It may be possible that some partner types display more severe aggressive behaviours while having a lower overall count of aggressions. For example, a partner may receive several more instances of headbutting or fencing while receiving less frequent orients – this aggression would appear less intense when considering only frequencies but may actually be more severe. Counts of each specific behaviour were recorded in the data gathered in this thesis, though their interpretation regarding intensity must first be preceded by further discussion of which behaviours are most severe.

4.8 40F04 *dsx* Neurons Influence Aggression but Not Grooming

Use of split-*Gal4* hemidriviers confirmed 40F04 neurons involved in female aggression do express *dsx* (Shirangi et al. 2016). This in combination with the lack of aggression observed in males with the same hemidriver combination further strengthens the role of *dsx* in developing sex-specific aggression neural circuitry.

Interestingly, restricted expression of *dTRPA1* to only those 40F04 neurons expressing *dsx* alleviated the head grooming phenotype observed in 40F04-*Gal4/+;UAS-dTRPA1/+* females and males. This confirms that the neurons controlling the two observed phenotypes are indeed different, and that the neurons inciting head grooming are not *dsx* expressing. Quality confocal microscope images would be highly useful in determining which of the 40F04 neurons are *dsx*-expressing, and therefore potentially required for the aggression phenotype. This would also inform which of the 40F04 are *not dsx*-expressing, and therefore potentially necessary for the head grooming phenotype.

Males expressing *dTRPA1* in 40F04-*dsx* neurons did not display either aggressive or grooming phenotypes but were observed to court wildtype *D. melanogaster* males. Use of proper controls is required for quantification and comparison to determine if this effect is a genuine result of the neural manipulation. It is entirely possible that these males court their partner males simply due to the non-discriminatory courtship criteria typical of males. However, if formal experimentation confirms an effect of this neural manipulation on inciting courtship behaviours in males, this would be consistent with previous observations (Rezával et al. 2016). More specifically, 40F04 neurons may control initiation of aggression in females and initiation of courtship in males. However, if this were the case, one would expect 40F04-*Gal4/+;UAS-dTRPA1/+* males to also display courtship, which was not observed. The absence of courtship in 40F04-*Gal4/+;UAS-dTRPA1/+* males may be explained by their excessive grooming. Hyperactivation of all male 40F04 neurons may produce signals encouraging both grooming and courtship, but the grooming signal takes precedence over that of the courtship signal. Refining the neural subset to 40F04-*dsx* neurons, and thus alleviating the grooming signal, is sufficient to unmask the courtship signal. It is possible that hyperactivating an even smaller subset of neurons will subdivide the male behavior into aggression vs. courtship, with courtship taking precedence over aggression when both sets of neurons are activated. Such “suppression hierarchies” have been identified in other behaviours such as grooming (Seeds et al. 2014).

It is surprising that females have aggression take precedence over grooming, while males prioritize grooming over courting. In females, hyperactivation of all 40F04 neurons may produce signals encouraging both aggression and grooming, with the aggression signal

overpowering that of the grooming signal. Given the appropriate stimuli, 40F04-*Gal4/+;UAS-dTRPA1/+* females will display aggression in place of grooming behaviours. In the absence of stimuli inciting aggression, the grooming signal is allowed to produce its phenotype. Again, restriction of *dTRPA1* expression to 40F04-*dsx* neurons alleviates the grooming signal, producing only aggressive behaviours. However, such an interpretation is dependent on formal experimental confirmation of the initial observation.

4.9 Future Work

The identification of the MB and alpha / beta lobes of the MB as effectors of female receptivity brings about several new questions warranting investigation. Firstly, similar manipulations of areas both downstream and upstream of the MB may be conducted to evaluate their potential roles in female receptivity circuitry. Similarly, assessment of which neurotransmitters are necessary for the MB to produce its effects on female receptivity would also aid in fleshing out the fine details of this circuit. As mentioned previously, it may also be worthwhile to re-investigate the effect of individual MB lobes, as use new *Gal4* or split-*Gal4* drivers may express in different subsets of neurons within the lobes and reveal an effect not shown by the drivers used here.

Understanding the influence of SIFamide on female receptivity would benefit from assessment of its roles in other areas of the brain including the alpha / beta and alpha prime / beta prime lobes of the MB, as well as areas of the protocerebrum such as the fan-shaped body, ellipsoid body, and the protocerebral bridge. Assessment of SIFamide receptor RNAi efficacy would also help in interpreting data presented here.

Additional experiments are also required to determine the aggression inducing stimuli in 40F04-*Gal4/+;UAS-dTRPA1/+* females. One such experiment includes the use of headless flies genetically altered to lack chemical cue producing oenocytes (Billeter et al. 2009), and perfumed with either female or male chemical cues. However, not all chemical cues are produced by the oenocytes, so this experiment would not be a perfect assessment of the potential roles of chemical cues in eliciting aggression. Future studies could also determine which neurotransmitters are necessary for 40F04 neurons to propagate their aggression and

grooming inducing signals. Due to the role of *dsx* neurons in influencing female post mating response, 40F04 neurons may also be assessed for roles in mediating female post-mating specific aggression.

4.10 Conclusions

I have demonstrated that manipulation of signaling within the MB, and specifically the alpha / beta lobes of the MB, reduces female receptivity to courting males. Similar manipulations of other subregions of the MB did not produce such changes, but cannot be definitively ruled out as influencers of this behaviour. Similarly, MB and MB lobe-specific knockdown of the SIFamide receptor also did not produce behavioural changes, though further investigation may be warranted. Finally, I have shown that hyperactivation of a subset of *dsx* neurons occupying the pC1 and pC2 clusters results in high amounts of female-specific aggression. I have characterized the behavioural phenotype in a number of contexts and have made progress in determining the precise stimuli triggering aggressive behaviours. Use of these females with genetically induced aggression will provide a novel and highly repeatable means of studying female aggression in a variety of contexts not possible before this discovery.

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